

# Microbiology

MICHAEL J. PELCZAR, JR.

E.C.S. CHAN

NOEL R. KRIEG



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**MICHAEL J. PELCZAR, JR.**

Professor Emeritus  
University of Maryland

**E. C. S. CHAN**

Associate Professor of Microbiology  
McGill University

**NOEL R. KRIEG**

Alumni Distinguished Professor of Microbiology  
Virginia Polytechnic Institute and State University

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# **PART ONE**

## **INTRODUCTION TO MICROBIOLOGY**



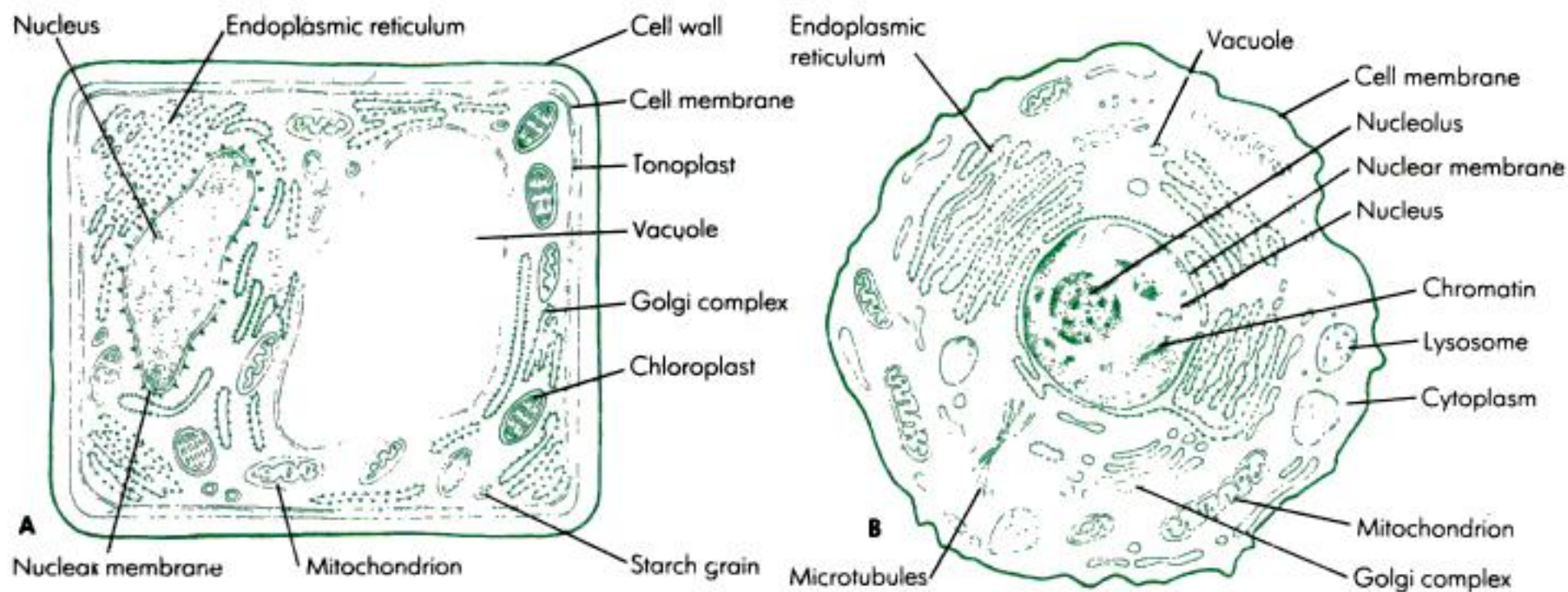


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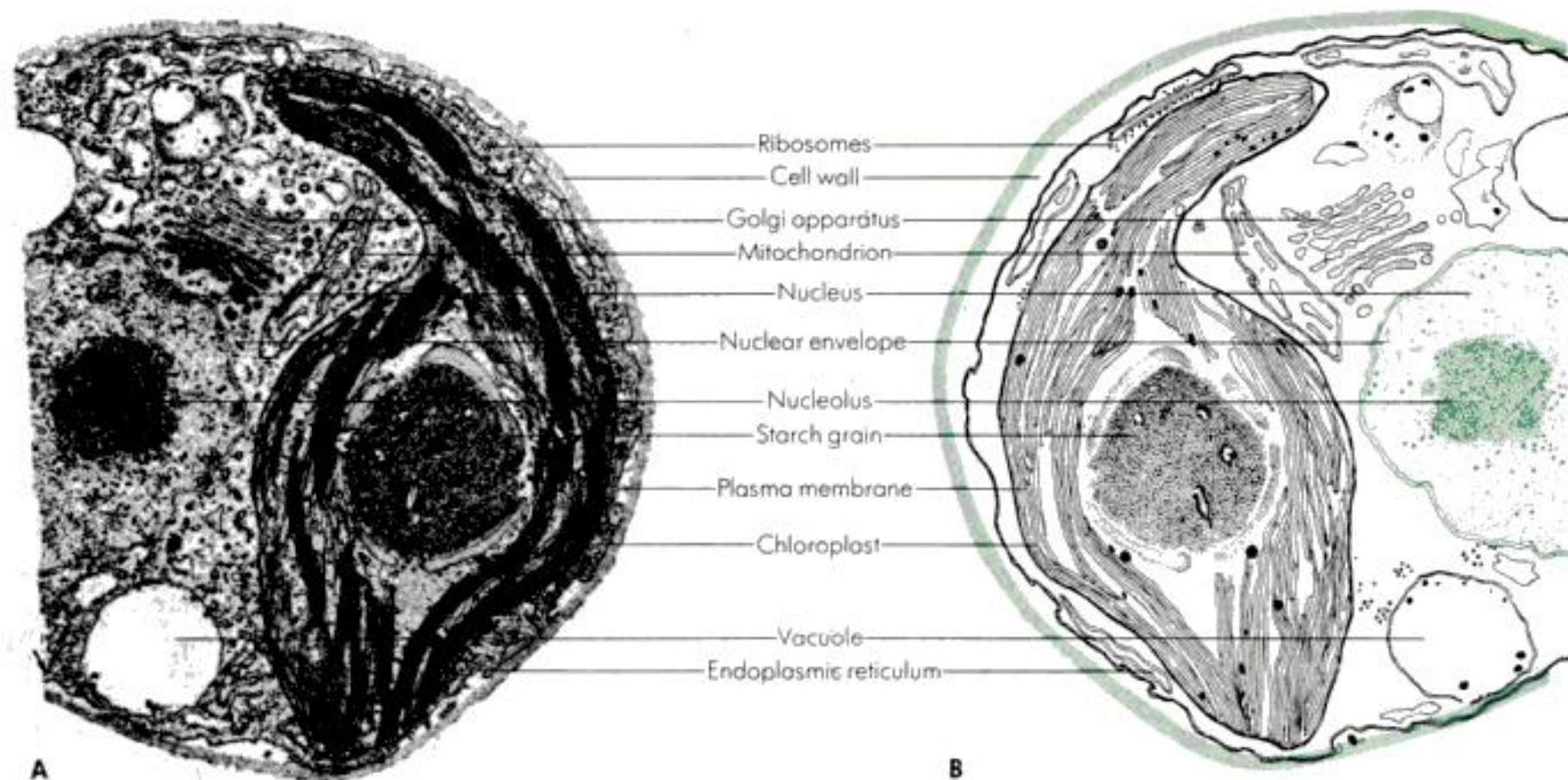
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**Figure 1-2.** Generalized diagram of typical cell structures. (A) Plant cell. (B) Animal cell. (C) Bacterial cell. (Erwin F. Lessel, illustrator.)



**Figure 1-3.** (A) Electron micrograph of the alga *Chlamydomonas reinhardtii* (X15,000), a eucaryotic cell. (Courtesy of George E. Palade, The Rockefeller University, by permission of Holt, New York, publishers of Ariel G. Leowy and Philip Seikovitz, *Cell Structure and Function*, 1969.) (B) Schematic representation of (A). (Erwin F. Lessel; illustrator.)



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**Table 1-1.** Features Distinguishing Prokaryotic from Eukaryotic Cells

Feature	Prokaryotic Cells	Eukaryotic Cells
Groups where found as unit of structure	Bacteria	Algae, fungi, protozoa, plants, and animals
Size range of organism	1–2 by 1–4 $\mu\text{m}$ or less	Greater than 5 $\mu\text{m}$ in width or diameter
Genetic system		
Location	Nucleoid, chromatin body, or nuclear material	Nucleus, mitochondria, chloroplasts
Structure of nucleus	Not bounded by nuclear membrane; one circular chromosome	Bounded by nuclear membrane; more than one chromosome
	Chromosome does not contain histones; no mitotic division	Chromosomes have histones; mitotic nuclear division
	Nucleolus absent; functionally related genes may be clustered	Nucleolus present; functionally related genes not clustered
Sexuality	Zygote nature is merozygotic (partial diploid)	Zygote is diploid
Cytoplasmic nature and structures		
Cytoplasmic streaming	Absent	Present
Pinocytosis	Absent	Present
Gas vacuoles	Can be present	Absent
Mesosome	Present	Absent
Ribosomes	70S,* distributed in the cytoplasm	80S arrayed on membranes as in endoplasmic reticulum; 70S in mitochondria and chloroplasts
Mitochondria	Absent	Present
Chloroplasts	Absent	May be present
Golgi structures	Absent	Present
Endoplasmic reticulum	Absent	Present
Membrane-bound (true) vacuoles	Absent	Present
Outer cell structures		
Cytoplasmic membranes	Generally do not contain sterols; contain part of respiratory and, in some, photosynthetic machinery	Sterols present; do not carry out respiration and photosynthesis
Cell wall	Peptidoglycan (murein or mucopeptide) as component	Absence of peptidoglycan
Locomotor organelles	Simple fibril	Multifibrilled with "9 + 2" microtubules

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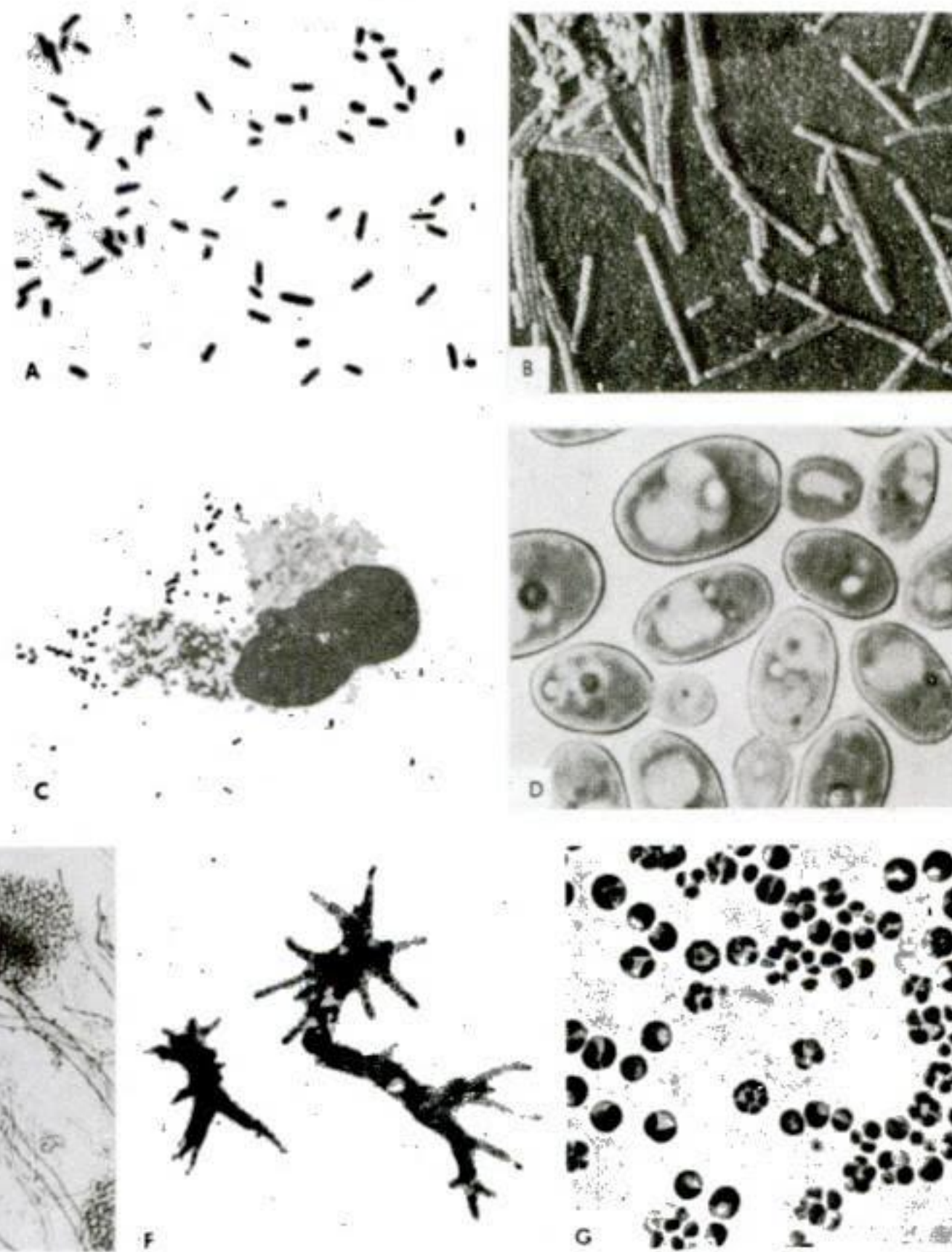
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**Figure 1-7.** Morphological features of various groups of microorganisms. (Note that this illustration is only intended to convey the impression of morphological diversity. No size relationship between groups can be obtained from it. The wide range in microbial sizes does not permit both constancy in magnification and showing of meaningful morphological details at the same time.) (A) *Escherichia coli* (X1,000). (B) Tobacco mosaic virus (X100,000). (Hitachi, Ltd., Tokyo.) (C) *Rickettsia tsutsugamushi* in cytoplasm of infected cell (X940). (N.J. Kramis and The Rocky Mountain Laboratory, U.S. Public Health Service.) (D) *Candida utilis* (X2,000 approx.). (Courtesy of G. Svihla, J. L. Dainko, and F. Schlenk, *J. Bacteriol.* 85:399, 1963.) (E) *Aspergillus* sp. (Courtesy of Douglas F. Lawson.) (F) *Amoeba*. (Carolina Biological Supply Co.) (G) *Chlorella infusionum* (X1,000). (Courtesy of Robert W. Krauss.)

mushrooms and puffballs. We are particularly interested in those organisms commonly called molds, the mildews, the yeasts, and the plant pathogens known as rusts. True fungi are composed of filaments and masses of cells which make up the body of the organism, known as a **mycelium**. Fungi reproduce by fission, by budding, or by means of spores borne on fruiting structures that are quite distinctive for certain species.

Some morphological and characteristic features of these various microbial groups are shown in Fig. 1-7 and Table 1-2.

Microbiologists may specialize in the study of certain groups of microorganisms. Strictly speaking, **bacteriology** is the study of bacteria, but the term is





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## QUESTIONS

- 1 List the characteristics common to all biological systems.
- 2 Why are microorganisms useful as subjects for research in the field of biology?
- 3 Explain why a knowledge of microbiology is useful in understanding life processes in higher plants and animals.
- 4 How did the term protists arise? What organisms do we refer to by use of this term? What is the difference between lower protists and higher protists?
- 5 Discuss the differences between procaryotic and eucaryotic cells.
- 6 How do viruses differ from other microorganisms?
- 7 What is the basis of the five-kingdom classification scheme according to Whittaker? Give a reason why it is so widely accepted in the biological community.
- 8 Discuss the place of microorganisms in Whittaker's five-kingdom classification scheme.
- 9 Why is *Bergey's Manual of Systematic Bacteriology* so important to bacteriologists?
- 10 Where are microorganisms found in nature? How may they be transferred from place to place?
- 11 Name several applied areas of microbiology. Describe the importance of microorganisms in each of these applied fields.

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water with great attention, especially those little animals appearing to me ten thousand times less than those . . . which may be perceived in the water with the naked eye.

He described his little animals in great detail, leaving little doubt that he saw bacteria, fungi, and many forms of protozoa. For example, he reported that on June 16, 1675, while examining well water into which he had put a whole pepper the day before:

I discovered, in a tiny drop of water, incredibly many very little animalcules, and these of divers sorts and sizes. They moved with bendings, as an eel always swims with its head in front, and never tail first, yet these animalcules swam as well backwards as forwards, though their motion was very slow.

His enthusiastic letters were read with interest by the British scientists, but the importance of his discoveries evidently went unappreciated. The talents and astuteness of this remarkable man can best be appreciated by reading Dobell's biography of Leeuwenhoek.

Before the time of Pasteur, microorganisms were studied mainly to satisfy curiosity concerning their characteristics and their relationships to higher living forms, without awareness of their importance in fermentation and disease.

## **SPONTANEOUS GENERATION VERSUS BIOGENESIS**

The discovery of microbes spurred interest in the origin of living things, and argument and speculation grew. As far as human beings were concerned, the Greek explanation that the goddess Gaea was able to create people from stones and other inanimate objects had been largely discarded. But even the astute Aristotle (384–322 B.C.) taught that animals might originate spontaneously from the soil, plants, or other unlike animals, and his influence was still strongly felt in the seventeenth century. About 40 B.C., Virgil (70–19 B.C.) gave directions for the artificial propagation of bees. This was but one of many fanciful tales of a similar nature that persisted into the seventeenth century. For example, it was accepted as a fact that maggots could be produced by exposing meat to warmth and air, but Francesco Redi (1626–1697) doubted this. Proof that his skepticism was well founded came from an experiment in which he placed meat in a jar covered with gauze. Attracted by the odor of the meat, flies laid eggs on the covering, and from the eggs maggots developed. Hence the experiment established the fact that the origin of the maggots was the flies and not the meat. This experiment and others involving mice and scorpions appear to have settled the matter so far as these forms of life were concerned. But microbes were another matter; surely such minute creatures needed no parents!

There appeared champions for and challengers of the theory that living things can originate spontaneously, each with a new and sometimes fantastic explanation or bit of experimental evidence. In 1749, while experimenting with meat exposed to hot ashes, John Needham (1713–1781) observed the appearance of organisms not present at the start of the experiment and concluded that the bacteria originated from the meat. About the same time, Lazzaro Spallanzani (1729–1799) boiled beef broth for an hour and then sealed the flasks. No microbes appeared following incubation. But his results, confirmed in repeated

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and then injected them into other animals to see if these became infected and developed clinical symptoms of anthrax. From these experimentally infected animals he isolated microbes like those he had originally found in sheep that died of anthrax. This was the first time a bacterium had been proved to be the cause of an animal disease. (Pebrine is caused by a protozoan rather than by a bacterium.) This series of observations led to the establishment of **Koch's postulates**, which provided guidelines to identify the causative agent of an infectious disease. Koch's postulates are: (1) A specific organism can always be found in association with a given disease. (2) The organism can be isolated and grown in pure culture in the laboratory. (3) The pure culture will produce the disease when inoculated into a susceptible animal. (4) It is possible to recover the organism in pure culture from the experimentally infected animal.

## LABORATORY TECHNIQUES AND PURE CULTURES

As we have previously stated, microorganisms occur in nature in extremely large populations made up of many different species. In order to study the characteristics of a particular species it is first necessary to separate it from all other species. Laboratory procedures have been developed that make it possible to isolate microorganisms representing each species and to grow (cultivate) each of the species separately. The growth of a mass of cells of the same species in a laboratory vessel (such as a test tube) is called a **pure culture**.

Pure cultures of bacteria were first obtained by Joseph Lister in 1878 using serial dilutions in liquid media. With a specially constructed syringe he diluted a fluid (probably milk) containing a mixture of bacteria until a single organism was delivered into a container of sterile milk. After incubation, bacteria in this container were of a single kind, identical to the parent cell. Lister named the organism *Bacterium lactis*.

Meanwhile Koch was carefully refining methods for the study of bacteria. He found that by smearing bacteria on a glass slide and adding certain dyes to them, individual cells could be seen more clearly with the microscope. He added gelatin and other solidifying materials such as agar to media in order to obtain isolated growths of organisms known as **colonies**, each of which contained millions of individual bacterial cells packed tightly together. From these colonies, pure cultures could be transferred to other media. The development of a liquefiable solid-culture medium was of fundamental importance.

Using techniques he had devised, Koch studied with painstaking care material taken from patients with pulmonary tuberculosis. After performing a series of rigid tests, as he had done with the anthrax bacillus, he announced the discovery of the microorganism that causes tuberculosis.

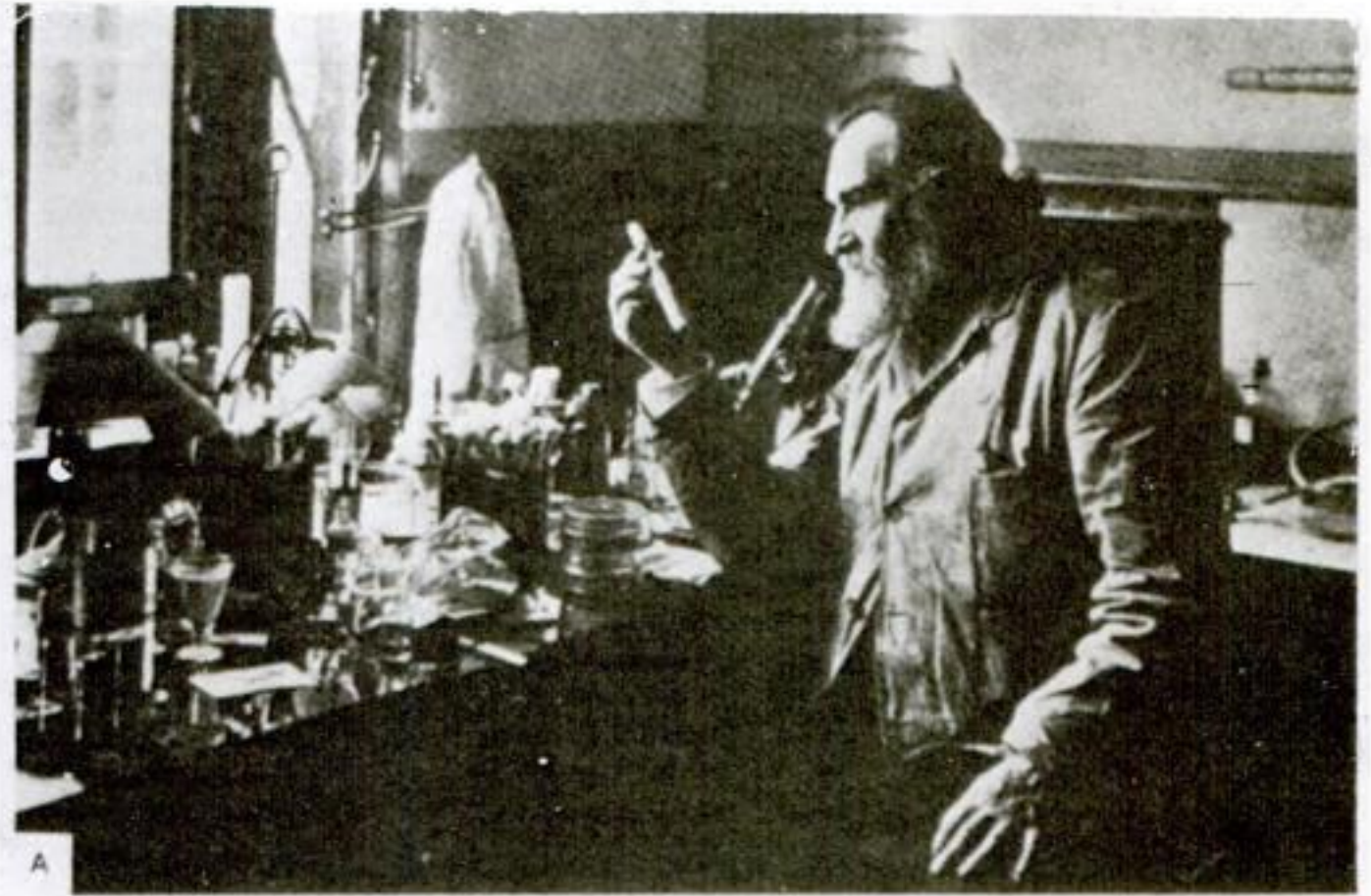
The importance of pure cultures to the development of the science of microbiology cannot be overestimated, since by using pure-culture techniques the microorganisms responsible for many infections, certain fermentations, nitrogen fixation in soil, and other activities were isolated and identified. However, strict adherence to pure-culture techniques and Koch's postulates sometimes led investigators up dead-end streets. Early investigators did not know about viruses, nor did they know about the cooperation of two or more microorganisms in causing disease or in bringing about a desirable fermentation such as we find in the ripening of cheese. Today we are as much interested in mixed microbial

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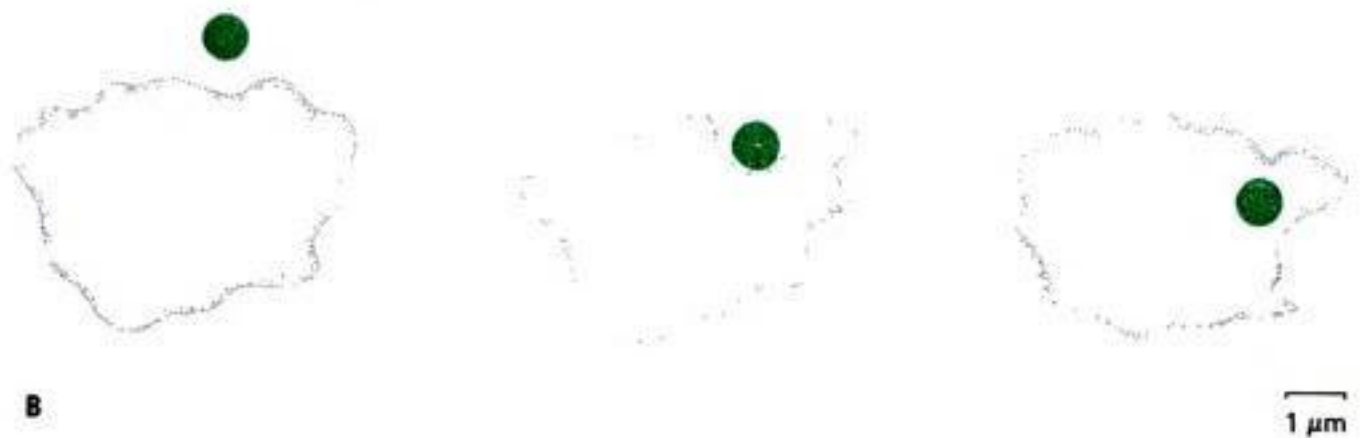
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**Figure 2-8.** (A) Elie Metchnikoff was the first person to recognize the role of phagocytes in combating bacterial infections. (Courtesy of René Dubos.) (B) The process of phagocytosis, the ingestion of particulate matter by certain cells, shown in three steps. Phagocytosis is a natural defense mechanism against disease. (Erwin F. Lessel, illustrator.)



microbiology because the infant science grew to adolescence during those years. As shown in Table 2-1, most of the causative agents of bacterial diseases that had plagued the world for centuries were isolated and identified.

The students of Koch and Pasteur continued to discover the causative agents of diseases and new methods for diagnosis; e.g., the Widal test for typhoid fever and the Wassermann test for syphilis made diagnosis of these diseases accurate and quick.

In the 1860s, while all these things were happening on the continent of Europe, an English surgeon, Joseph Lister, was trying to combat the microbes that caused postoperative and wound infections. Deaths from these infections were frequent in the nineteenth century. Disinfectants as such were unknown, but since carbolic acid would kill bacteria, Lister used a dilute solution of this acid to soak surgical dressings. Wounds protected in this way did not become infected, and healing took place rapidly. So remarkable was Lister's success that the technique was quickly accepted, and this antiseptic surgical practice established the principles of present-day aseptic ("without infection") techniques.

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Table 2-2. (continued)

Era	Investigator	Contribution
	August von Wassermann (1866–1925)	Introduced complement-fixation test for syphilis
	Martinus Willem Beijerinck (1851–1931)	Utilized principle of enrichment cultures; confirmed finding of first virus
	Frederick W. Twort (1877–1950) Felix H. d'Herelle (1873–1949)	{ Independently discovered bacteriophages, viruses that destroy bacteria
	Howard T. Ricketts (1871–1910)	Reported Rocky Mountain spotted fever transmitted by wood tick and Mexican typhus transmitted by body louse

### MICROBIOLOGY AND MODERN BIOLOGY: MOLECULAR BIOLOGY

As new laboratory techniques and experimental procedures were developed, our knowledge of the characteristics of microorganisms accumulated rapidly. Extensive information about the biochemical activities of microorganisms became available. An analysis of the data suggested that there was much in common among different microorganisms—the differences were likely to be variations on a major central biochemical pathway. At about the same time there was a growing recognition of the unity of the biochemical life processes in microorganisms and higher forms of life, including human beings. Consequently it became attractive to use microorganisms as a tool to explore fundamental life processes. Microorganisms offer numerous advantages for this kind of research: they reproduce (grow) very rapidly, they can be cultured (grown) in small or vast quantities conveniently and rapidly, their growth can be manipulated easily by chemical or physical means, and their cells can be broken apart and the contents separated into fractions of various particle sizes. These characteristics, as well as others, make microorganisms a very convenient research model for determining exactly how various life processes take place in terms of specific chemical reactions and the specific structures involved. Scientists from many disciplines recognized the usefulness of microorganisms as experimental models. Thus it was not surprising that physicists, geneticists, chemists, and biologists joined with microbiologists in what is now known as **molecular biology**. Salvador E. Luria (Fig. 2-9), professor of biology at the Massachusetts Institute

**Figure 2-9.** Salvador E. Luria, Professor of Biology at the Massachusetts Institute of Technology, was awarded the Nobel prize in 1969 for his research in molecular biology.



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## Chapter 3

# The Characterization, Classification, and Identification of Microorganisms

### OUTLINE

#### Major Characteristics of Microorganisms

Morphological Characteristics • Chemical Characteristics • Cultural Characteristics • Metabolic Characteristics • Antigenic Characteristics • Genetic Characteristics • Pathogenicity • Ecological Characteristics

#### Microbial Classification, Nomenclature, and Identification

Classification • Nomenclature • Identification • The Past and Present State of Bacterial Taxonomy

Characterization, classification, and identification are major objectives in all branches of the biological sciences.

Classification is a means of bringing order to the bewildering variety of organisms in nature. Once we learn the characteristics of an organism we can compare it with other organisms to discover similarities and differences. The human mind tends to arrange similar things together in groups and to distinguish these groups from one another.

In order to identify and classify microorganisms, we must first learn their **characteristics**. It is usually not feasible to study the characteristics of a single microorganism, because of its small size; therefore, we study the characteristics of a **culture**—a population of microorganisms. If we study the characteristics of a culture containing many microorganisms (usually millions or billions of cells of only one kind), it is as if we are studying the characteristics of a single organism.

As stated earlier, a culture that consists of a single kind of microorganism (one living species), regardless of the number of individuals, in an environment free of other living organisms is called a **pure culture**. (Strictly speaking, this is an **axenic culture**; however, microbiologists customarily refer to such a culture as a pure culture. In the strict, technical sense a pure culture is one grown from a single cell.)

Determining the characteristics of microorganisms is not only prerequisite for classification but is done for other reasons as well. As we have already pointed out, microorganisms play many important, indeed essential, roles in nature. It is therefore desirable to determine the characteristics of species that enable these activities to occur.

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For example, bacteria known as **bdellovibrios** are predatory on other bacteria, and viruses called **bacteriophages** can infect and destroy bacterial cells.

## Ecological Characteristics

The habitat of a microorganism is important in characterizing that organism. For example, microorganisms normally found in marine environments generally differ from those in freshwater environments. The microbial population of the oral cavity differs from that of the intestinal tract. Some kinds of microorganisms are widely distributed in nature, but others may be restricted to a particular environment. The relation of an organism to its environment is often complex and may involve special characteristics of the organism that are not yet known.

## MICROBIAL CLASSIFICATION, NOMENCLATURE, AND IDENTIFICATION

Once the characteristics of microorganisms have been determined and appropriately catalogued, the process of classification can begin.

### Classification

In microbiology, taxa are initially constructed from **strains**. A strain is made up of all the descendents of a pure culture; it is usually a succession of cultures derived from an initial colony. Each strain has a specific history and designation.

### Taxonomic Groups (Taxa)

For example, strain ATCC 19554 is a strain of spirilla isolated originally from pond water in Blacksburg, Virginia in 1965 by Wells and Krieg, and cultures of this strain are maintained at the American Type Culture Collection (ATCC), Rockville, Maryland. Cultures of the same species that were isolated from other sources would be considered different strains.

The basic taxonomic group (taxon) is the **species**, i.e., a collection of strains having similar characteristics. Bacterial species consist of a special strain called the **type strain** together with all other strains that are considered sufficiently similar to the type strain as to warrant inclusion in the species. The type strain is the strain that is designated to be the permanent reference specimen for the species. Unfortunately, it is not always the strain that is most typical of all the strains included in the species, but it is the strain to which all other strains must be compared to see if they resemble it closely enough to belong to the species. Therefore, type strains are particularly important and special attention is given to their maintenance and preservation, particularly by national reference collections such as the ATCC in the United States or the National Collection of Type Cultures in England. Many other culture collections are maintained throughout the world.

In the definition just given for a bacterial species, the phrase "considered sufficiently similar to the type strain" indicates that the definition contains an element of subjectivity. In other words, the criteria which one taxonomist believes to constitute "sufficient similarity" may be quite different from those used by another taxonomist. At present there are no specific criteria for a bacterial species that are universally accepted. However, certain criteria based on DNA homology experiments (described later in this chapter) are probably more widely accepted today than any others and eventually may lead to a unifying concept for defining a species.

Just as a bacterial species is composed of a collection of similar strains, a

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**2 Ribosomal RNA homology experiments and ribosomal RNA oligonucleotide cataloging.** Two organisms may not be so closely related as to give a high level of DNA homology, yet they may still have some degree of relatedness. **Ribosomes**, the small granular-appearing structures within the cell which manufacture proteins, are composed of proteins and RNA. The ribosomal RNA (rRNA) is coded for by only a small fraction of the DNA molecule, the **rRNA cistrons**. In all bacteria so far studied, the nucleotide sequence of these rRNA genes has been found to be **highly conserved**; that is, during evolution, the nucleotide sequence has changed more slowly than that of the bulk of the DNA molecule. This means that even if two organisms are only distantly related and show no significant DNA homology, there still may be considerable similarity in the nucleotide sequences of their rRNA cistrons. The degree of similarity that exists can therefore be used as a measure of relatedness between organisms, but at a level beyond that of species (at the level of genus, family, order, etc.). RNA homology experiments and RNA oligonucleotide cataloging are two modern methods used to determine the degree of similarity between the rRNA cistrons of different organisms. The techniques are complex and are being used by only a few laboratories.

Classifications based on genetic relatedness come the closest to achieving the taxonomic goals of stability and predictability. Moreover, the data obtained for such classifications allow microbiologists to infer the way in which bacteria have evolved, so that the present-day bacterial genera and species can be arranged in a hierarchy that reflects their ancestral relationships, i.e., in a **phylogenetic** classification. Much of the work is still fragmentary, but some of the results, especially those obtained by Dr. C. R. Woese of the University of Illinois and his colleagues, have already revolutionized current thinking about how bacteria have evolved and how they are related to one another. In fact, it is now apparent that present-day bacteria evolved by at least two very different major routes from an early ancestral form and that they now comprise two very large groups: the **eubacteria** (which are the traditional, familiar ones that have received the most study) and the **archaeobacteria** (consisting of methane-producers, extreme halophiles, and thermoacidophiles). It has been proposed that these two groups be considered as two separate kingdoms of life, and, indeed, they do seem as distantly related to each other as they are to eucaryotic organisms. Although the kingdom question is still debatable, data obtained from rRNA oligonucleotide cataloging nevertheless make it clear that the archaeobacteria are separated from other bacteria by a great phylogenetic gulf (see Fig. 3-3).

## Nomenclature

Each species of microorganism has **only one officially accepted name**, by international agreement. This system provides for precise communication. If an organism were to be called *Escherichia coli* in one country and *Coprobacterium intestinale* in another, chaos would result. It would be difficult to know that the same organism was being studied.

The name of a species is merely a convenient label. It is not necessarily even descriptive, although some names are. For example, *Micrococcus luteus* means "yellow berry" in Latin, and *Proteus vulgaris* is Latin for "common organism of many shapes." Some species are named after persons: for example, *Escherichia coli*—the organism of the colon, named after Theodor Escherich (a German bacteriologist); or *Clostridium barkeri*—the spindle-shaped organism,

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- 6 (a) If two microorganisms have an identical mol% G + C value for their DNA, are they necessarily related? Explain.
- (b) If two microorganisms have very different mol% G + C values for their DNA, are they necessarily unrelated? Explain.
- 7 In DNA homology experiments, we directly compare the entire **genome** (all the DNA) of one organism with that of another organism. What are we comparing when we do rRNA homology experiments or rRNA oligonucleotide cataloging?
- 8 What advantages do rRNA homology experiments and rRNA oligonucleotide cataloging offer compared to DNA homology experiments?
- 9 What is the reason that each taxon has only one officially recognized name?
- 10 What function does the name of a bacterial species serve?
- 11 Give an example of a bacterial name and write it in its proper form.
- 12 What makes *Bergey's Manual* unique among microbiological publications?
- 13 What is the present philosophy of the editorial board of *Bergey's Manual* toward bacterial classification and what sorts of changes may occur in future editions?

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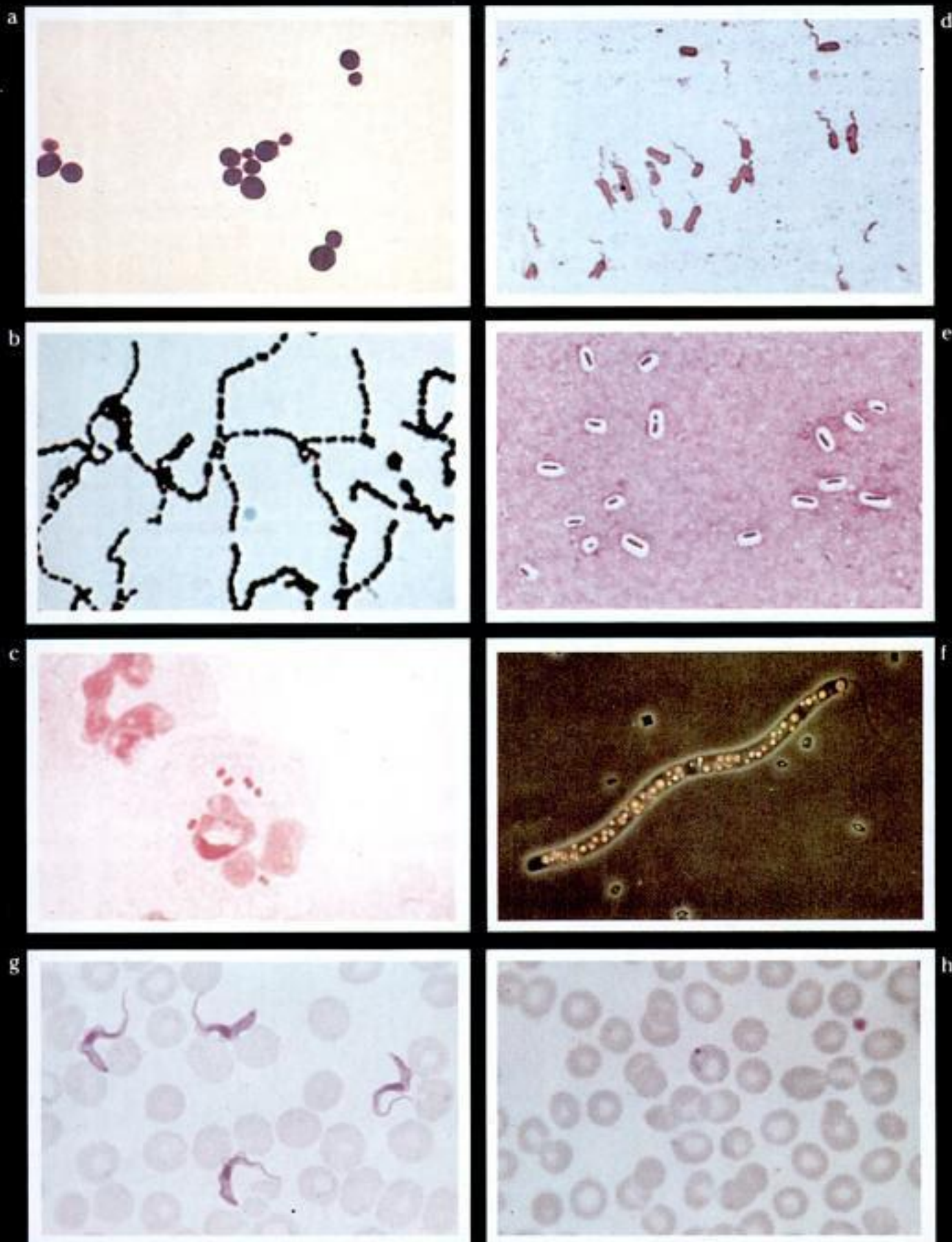
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# Microscopic preparations:

- a The yeast *Candida albicans* stained by the Gram method; the blue color indicates that it is a Gram-positive organism.
- b Gram stain of *Streptococcus pyrogenes* shows that this species is Gram-positive.
- c Gram stain of specimen combining *Neisseria gonorrhoeae*, the causative agent of gonorrhea. This bacterium is Gram-negative; the cells appear red. In this specimen the gonococci appear as small diplococci within a white blood cell.
- d Flagella stain of *Pseudomonas aeruginosa*. A special staining technique is required to demonstrate with light microscopy the presence of flagella on bacteria.
- e Capsule stain of *Klebsiella pneumoniae*, the bacterial cells appear dark within a clear envelope.
- f *Thiospirillum jenense* with sulfur globules (X1100).
- g *Trypanosoma cruzi*, causative agent of Chagas' disease as seen in a stained blood film.
- h *Plasmodium vivax*, causative agent of malaria. Stain of a blood film showing trophozoite in red blood cell.

(Figures a to e courtesy of Microbiology Service, Clinical Pathology Department, National Institutes of Health; Fig. f courtesy of R. L. Gherna, American Type Culture Collection; Figs. g and h courtesy of National Medical Audiovisual Center, Centers for Disease Control, Atlanta, Georgia.)

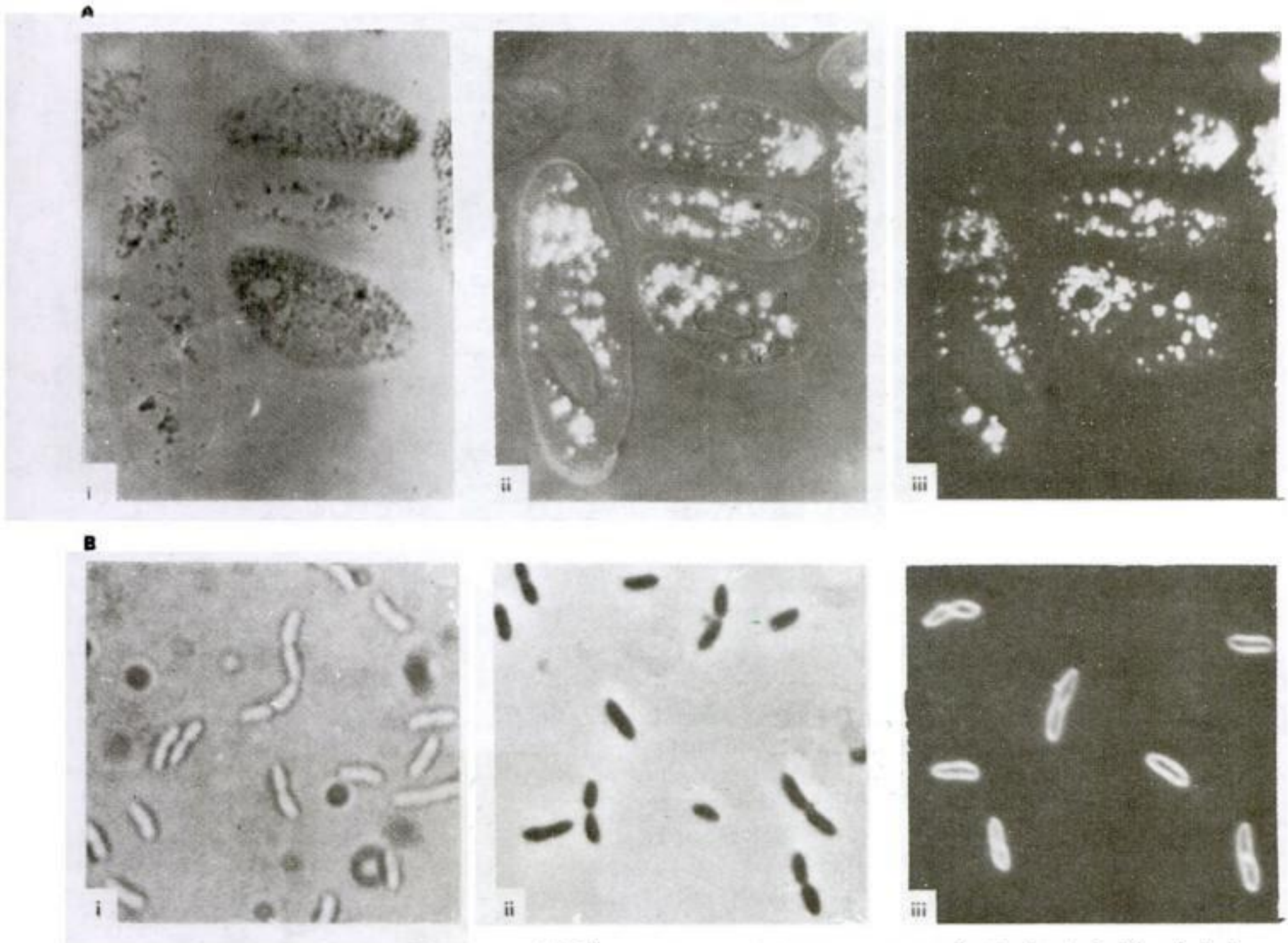


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**Figure 4-9.** (A) Phase-contrast microscopy compared with bright-field and dark-field microscopy. The same specimen of a protozoan as seen by each method: (i) bright-field; (ii) phase-contrast; (iii) dark-field. (Courtesy of O. W. Richards, Research Department, American Optical Company.) (B) Photomicrographs of living, unstained, rod-shaped cells of *Pseudomonas fluorescens*. The bacilli are 0.7 to 0.8  $\mu\text{m}$  in width. They can be seen only indistinctly by ordinary bright-field microscopy (i) but are readily visible by phase-contrast (ii) or dark-field microscopy (iii). (Courtesy N. R. Krieg.)

son of a specimen viewed by bright-field, dark-field, and phase-contrast microscopy is shown in Fig. 4-9.

### Transmission Electron Microscopy

Electron microscopy differs markedly and in many respects from the optical microscopic techniques. The electron microscope provides tremendous useful magnification, because of the much higher resolution obtainable with the ex-

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**Figure 4-15.** Electron micrograph of freeze-etched preparation of *Neisseria gonorrhoeae*. This bacterium measures approximately 0.6 to 1.0  $\mu\text{m}$  in diameter. (Courtesy of Ivan L. Roth.)

### Negative Staining

An electron-dense material such as phosphotungstic acid can be used as a "stain" to outline the object. The electron-opaque phosphotungstate does not penetrate structures but forms thick deposits in crevices (see Fig. 4-14). Fine detail of objects such as viruses or bacterial flagella can be seen by this technique.

### Ultrathin Sectioning

In order to make observations of intracellular structures, the material for examination must be extremely thin. An intact microbial cell is too thick to allow distinct visualization of its internal fine structure by electron microscopy. However, techniques are available for sectioning (slicing) a bacterial cell; for example, bacterial cells can be embedded in a plastic material and then this "block" can be cut into ultrathin slices, as thin as 60 nm. These slices are then prepared for microscopic examination. As you might expect, the slices will reveal cells sliced at different levels and at different angles. Improvement in contrast of structures is possible through use of special electron-microscope stains such as uranium and lanthanum salts.

### Freeze-Etching

Freeze-etching was developed to prepare sections of the specimen without resorting to the chemical treatment of the fixation process, which can produce artifacts. The specimen is sectioned while contained in a frozen block. Carbon replicas of these exposed surfaces are then prepared which reveal internal structures of the cell (see Fig. 4-15).

### Localization of Cell Constituents

Special techniques have been developed making it possible to locate chemical constituents of the cell. For example, thin sections of a cell can be treated with

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Examination of microorganisms in wet preparation is desirable in the following instances:

- 1 The morphology of spiral bacteria is greatly distorted when these bacteria are dried and stained; they should be examined in living condition. For example, in the examination of serous exudates suspected of containing the spirochete that causes syphilis, the wet preparations are examined by dark-field microscopy. This provides a sharp contrast between the organisms and the dark background. The normal arrangement of cells can also be better determined in a wet preparation.
- 2 The observation of bacteria to determine whether or not they are motile obviously requires that they be suspended in a liquid medium, free to move about.
- 3 To observe cytological changes occurring during cell division and to determine the rate at which the division occurs, the organisms must be examined in the living state (i.e., wet mount). Spore formation and germination must also be observed in living cells.
- 4 Some cell inclusion bodies, e.g., vacuoles and lipid material, can be observed readily by this method.

When wet preparations are examined by bright-field microscopy, it is extremely important to control the light source. The reason is that the lack of a stain makes the cells less distinctly visible; adjustment of the intensity of the light source can enhance their visibility. Partially closing the substage condenser diaphragm helps to increase contrast; however, some resolving power is lost. Dark-field and phase-contrast microscopy offer the distinct advantage of providing both high contrast and high resolving power for examination of unstained preparations.

### Fixed, Stained Smears

Fixed, stained preparations are most frequently used for the observation of the morphological characteristics of bacteria. The advantages of this procedure are that (1) the cells are made more clearly visible after they are colored, and (2) differences between cells of different species and within the same species can be demonstrated by use of appropriate staining solutions (differential or selective staining).

The essential steps in the preparation of a fixed, stained smear are (1) preparation of the film or smear, (2) fixation, and (3) application of one or more staining solutions.

### Microbiological Stains

A large number of colored organic compounds (dyes) are available for staining microorganisms. These compounds are generally rather complex in terms of molecular structure. On this basis they may be classified into groups such as triphenylmethane dyes, oxazine dyes, and thiazine dyes.

A more practical classification for the cytologist is one based on the chemical behavior of the dye; namely, acid, basic, or neutral. An acid (or anionic) dye is one in which the charge on the dye ion is negative; a basic (or cationic) dye is one in which the charge carried by the dye ion is positive. A neutral dye is a complex salt of a dye acid with a dye base, e.g., eosinate of methylene blue. Acid dyes generally stain basic cell components, and basic dyes generally stain acidic cell components.

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functional properties of the bacterial cell. This area of research studied by biologists is sometimes referred to as **biochemical cytology**.

## THE SIZE, SHAPE, AND ARRANGEMENT OF BACTERIAL CELLS

### Size

Bacteria are very small, most being approximately 0.5 to 1.0  $\mu\text{m}$  in diameter. An important consequence of the small size of microorganisms is that the **surface area/volume ratio** of bacteria is exceedingly high compared to the same ratio for larger organisms of similar shape (Table 5-1). A relatively large surface through which nutrients can enter (or waste products leave) compared to a small volume of cell substance to be nourished accounts for the unusually high rate of growth and metabolism of bacteria. Moreover, because of the high surface area/volume ratio, the mass of cell substance to be nourished is very close to the surface; therefore, no circulatory mechanism is needed to distribute the nutrients that are taken in, and there is thought to be little or no cytoplasmic movement within a bacterial cell. Despite these advantages, a high surface area/volume ratio limits the size of bacteria to microscopic dimensions.

### Shape and Arrangement

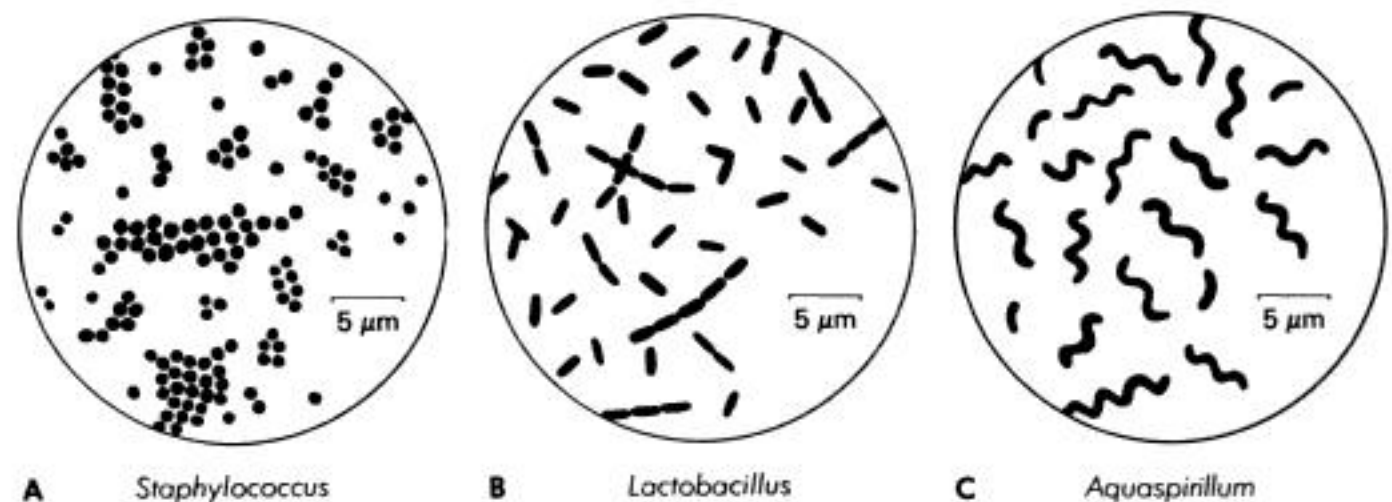
The shape of a bacterium is governed by its rigid cell wall; however, exactly what attribute of this rigid material determines that a cell will have a particular shape is not yet understood. Typical bacterial cells are spherical (**cocci**; singular, **coccus**); straight rods (**bacilli**; singular, **bacillus**); or rods that are helically curved (**spirilla**; singular, **spirillum**) as illustrated in Fig. 5-1. Although most bacterial species have cells that are of a fairly constant and characteristic shape, some have cells that are **pleomorphic**, i.e., that can exhibit a variety of shapes (Fig. 5-2).

**Table 5-1.** Comparison of the Surface Area/Volume Ratio of Spherical Organisms of Different Sizes\*

Diameter of Sphere, $\mu\text{m}$	Surface Area, $\mu\text{m}^2$ ( $4\pi r^2$ )	Volume, $\mu\text{m}^3$ ( $4\pi r^3/3$ )	Surface Area/ Volume, $\mu\text{m}^{-1}$ ( $3/r$ )
1 $\mu\text{m}$	3.1	0.52	6
1,000 $\mu\text{m}$	$3.1 \times 10^6$	$5.2 \times 10^8$	0.006
1,000,000 $\mu\text{m}$	$3.1 \times 10^{12}$	$5.2 \times 10^{17}$	0.000006

\* For a given volume, the geometrical shape that has the smallest surface area/volume ratio is a sphere; i.e., if two organisms have the same volume, one being spherical and the other cylindrical, the cylindrical organism has the greater surface area/volume ratio.

**Figure 5-1.** Bacteria are generally either (A) spherical (cocci); (B) rodlike (rods or bacilli); or (C) helical (spirilla). However, there are many modifications of these three basic forms. (Erwin F. Lessel, illustrator.)



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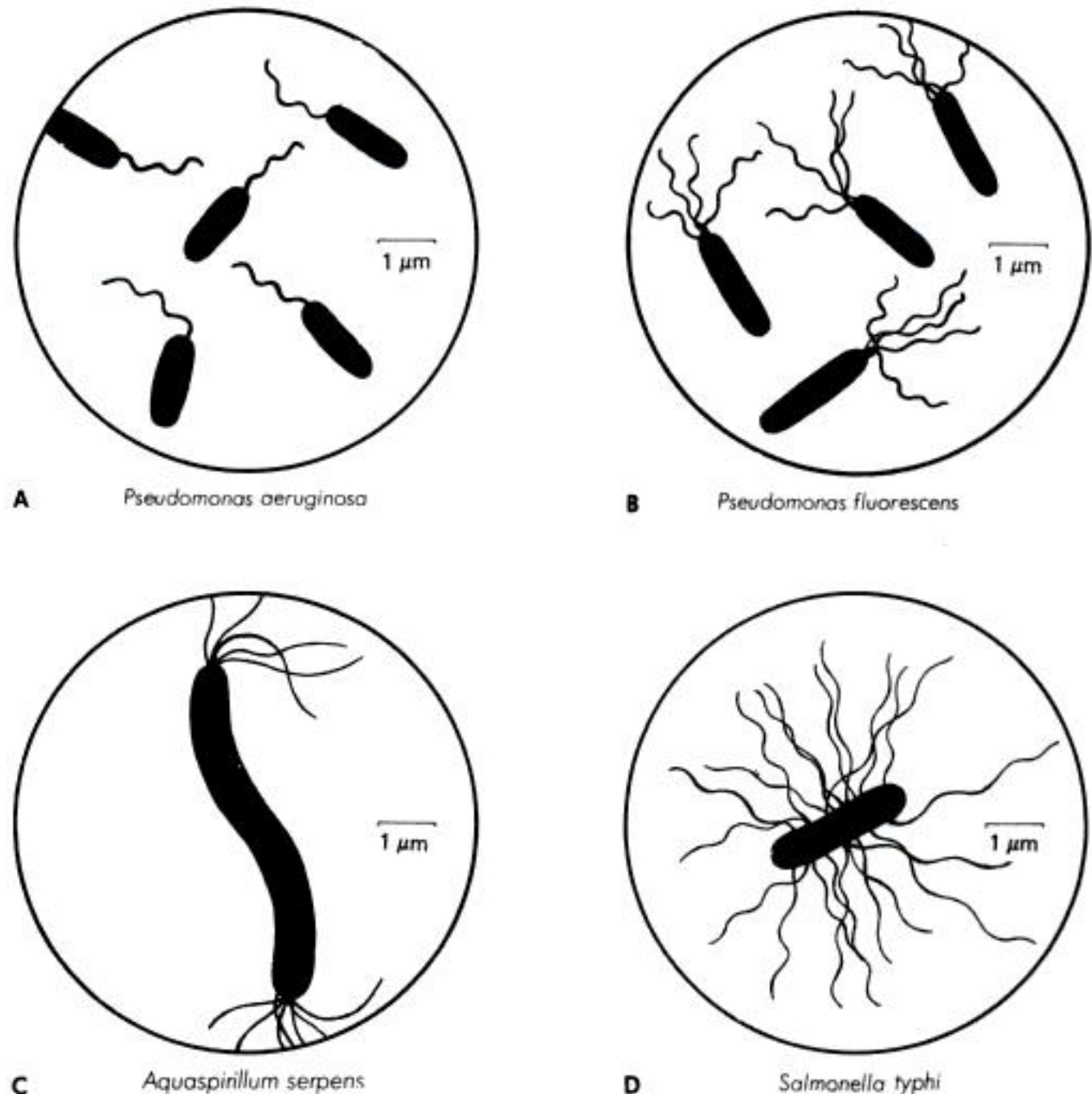
## STRUCTURES EXTERNAL TO THE CELL WALL

### Flagella and Motility

Bacterial **flagella** (singular, **flagellum**) are hairlike, helical appendages that protrude through the cell wall and are responsible for swimming motility. They are much thinner than the flagella or cilia of eucaryotes, being 0.01 to 0.02  $\mu\text{m}$  in diameter, and they are also much simpler in structure. Their location on the cell varies depending on the bacterial species and may be **polar** (at one or both ends of the bacterium) or **lateral** (along the sides of the bacterium). Some arrangements of bacterial flagella are shown in Fig. 5-11. A flagellum is composed of three parts (Fig. 5-12): a **basal body** associated with the cytoplasmic membrane and cell wall, a short **hook**, and a helical **filament** which is usually several times as long as the cell. Some Gram-negative bacteria have a sheath surrounding the flagellum; this sheath is continuous with the outer membrane of the Gram-negative cell wall. The chemical composition of the basal body is unknown, but the hook and filament are composed of protein subunits (monomers) arranged in a helical fashion. The protein of the filament is known as **flagellin**.

Unlike a hair, a flagellum grows at its tip rather than at the base. Flagellin monomers synthesized within the cell are believed to pass along the hollow center of the flagellum and are added to the distal end of the filament.

**Figure 5-11.** Drawings of various arrangements of bacterial flagella. (A) Monotrichous; a single polar flagellum. (B) Lophotrichous; a cluster of polar flagella. (C) Amphitrichous; flagella, either single or clusters, at both cell poles. (D) Peritrichous; surrounded by lateral flagella. (Erwin F. Lessel, illustrator.)



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## Pili (Fimbriae)

**Pili** (singular, **pilus**) are hollow, nonhelical, filamentous appendages that are thinner, shorter, and more numerous than flagella (Fig. 5-15). They do not function in motility, since they are found on nonmotile as well as motile species. There are, however, several functions associated with different types of pili. One type, known as the F pilus (or sex pilus), serves as the port of entry of genetic material during bacterial mating (see Chap. 12). Some pili play a major role in human infection by allowing pathogenic bacteria to attach to epithelial cells lining the respiratory, intestinal, or genitourinary tracts. This attachment prevents the bacteria from being washed away by the flow of mucous or body fluids and permits the infection to be established.

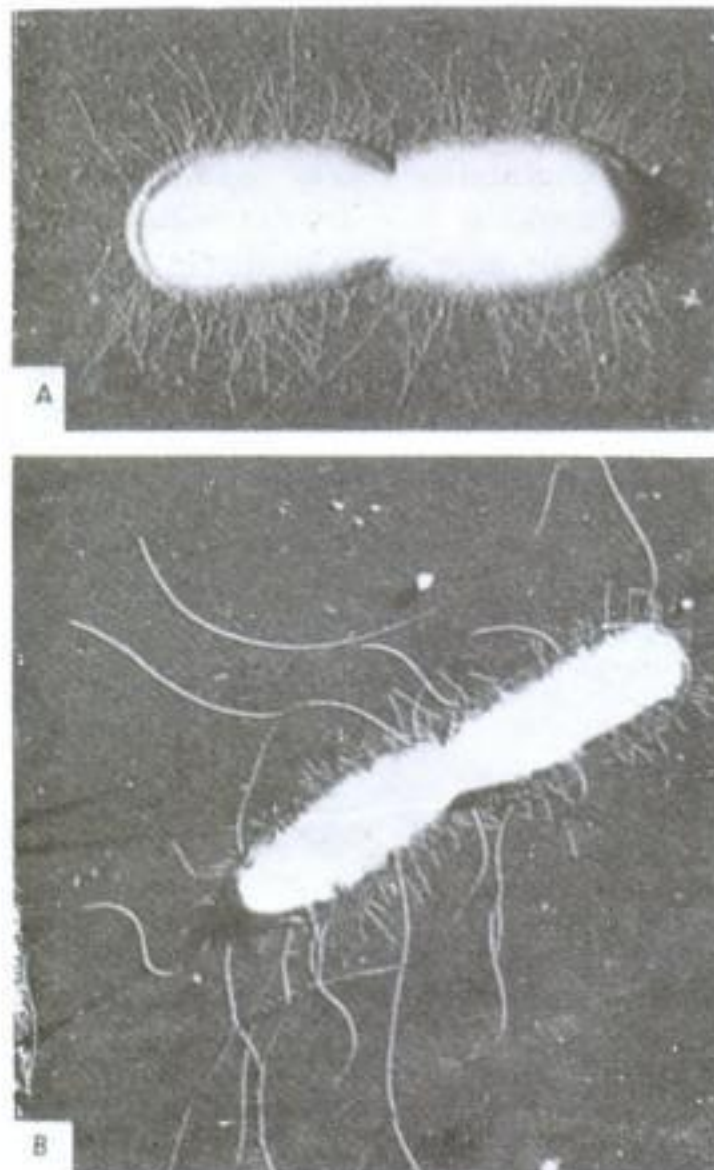
## Capsules

Some bacterial cells are surrounded by a viscous substance forming a covering layer or envelope around the cell wall. If this layer can be visualized by light microscopy using special staining methods, it is termed a **capsule**. If the layer is too thin to be seen by light microscopy it is termed a **microcapsule**; if it is so abundant that many cells are embedded in a common matrix, the material is called **slime**.

By light microscopy, capsules appear to be amorphous gelatinous areas surrounding a cell (Fig. 5-16A); however, special techniques designed to preserve delicate structures for observation by electron microscopy have revealed that capsules consist of a mesh or network of fine strands (Fig. 5-16B).

In many instances capsular material is not highly water-soluble and therefore does not readily diffuse away from the cells that produce it. In other instances

**Figure 5-15.** Fimbriated bacteria. (A) *Shigella flexneri*: dividing bacilli with numerous fimbriae surrounding the cells (X20,000). (B) *Salmonella typhi*: dividing bacilli with numerous fimbriae and a few flagella (the very long appendages) (X12,500). (Courtesy of J. P. Duguid and J. F. Wilkinson and The Society of General Microbiology: Symposium XI, 1961.)



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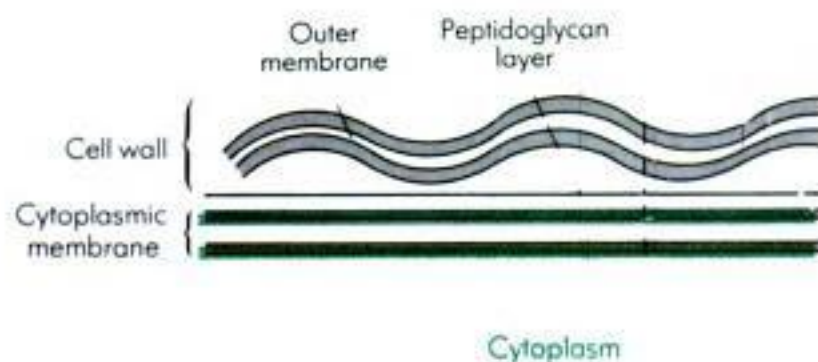
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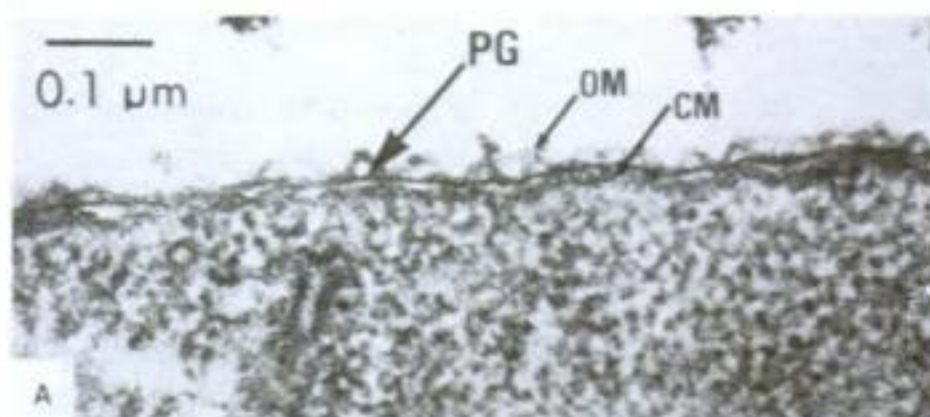


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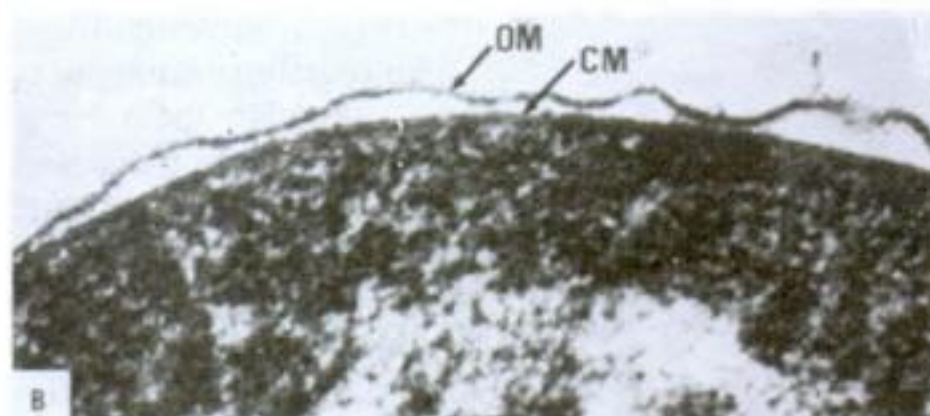


B

**Figure 5-19.** Schematic interpretation of cell walls of eubacteria from electron-microscope observations. (A) Gram-positive bacteria, showing thick wall consisting mainly of peptidoglycan. Although the wall is often homogeneous in appearance, in some bacteria it may consist of several layers. (B) Gram-negative bacteria, showing outer membrane and thin peptidoglycan layer. (Courtesy of A. I. Laskin and H. A. Lechevalier (eds.), *Handbook of Microbiology*, CRC Press, Inc., Cleveland, 1974.)



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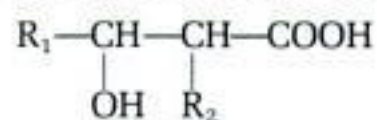


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**Figure 5-20.** (A) Thin section of *Aquaspirillum serpens* showing the wavy outer membrane (OM), the peptidoglycan layer (PG), and the cytoplasmic membrane (CM). (B) Companion preparation of a spheroplast formed by treatment of the cells with a chelating agent and lysozyme. The peptidoglycan layer is missing. (From R. G. E. Murray, P. Steed and H. E. Elson, *Can J Microbiol* 11:547, 1965.)

about 10 percent of the wall of Gram-negative bacteria. Other substances may occur in addition to peptidoglycan. For instance, the walls of *Streptococcus pyogenes* contain **polysaccharides** that are covalently linked to the peptidoglycan and which can be extracted with hot dilute hydrochloric acid. The walls of *Staphylococcus aureus* and *Streptococcus faecalis* contain **teichoic acids**—acidic polymers of ribitol phosphate or glycerol phosphate—which are covalently linked to peptidoglycan and which can be extracted with cold dilute acid. Teichoic acids bind magnesium ions, and there is some evidence that they help to protect bacteria from thermal injury by providing an accessible pool of these cations for stabilization of the cytoplasmic membrane. The walls of most Gram-positive bacteria contain very little lipid, but those of *Mycobacterium*, *Corynebacterium*, and certain other genera are exceptions, being rich in lipids called

These compounds have the following general structure:



where  $\text{R}_1$  and  $\text{R}_2$  are long hydrocarbon chains. The ability of mycobacteria to exhibit acid-fast staining (i.e., when stained, the cells cannot be decolorized

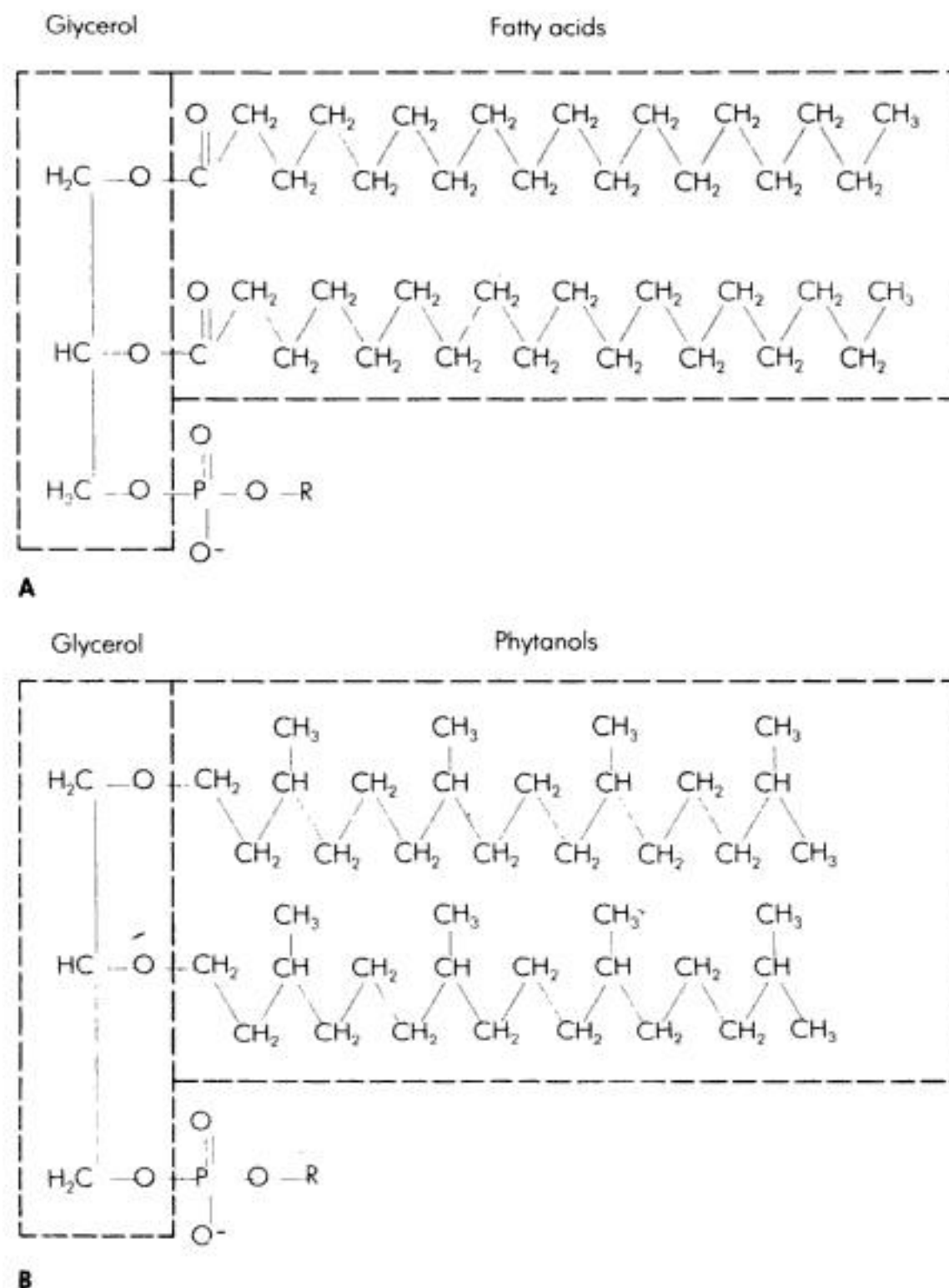
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**Figure 5-25.** (A) Example of a eubacterial phospholipid, showing two unbranched, long-chain fatty acids ester-linked to glycerol. (B) Example of an archaeobacterial phospholipid, showing two branched phytanol chains that are ether-linked to glycerol. (R is any of several compounds such as ethanolamine, choline, serine, inositol, or glycerol.)



membrane is an extremely important functional structure, and damage to it by physical or chemical agents can result in the death of the cell.

Proteins are synthesized within the cell, but some can pass across the cytoplasmic membrane barrier to the outside; examples of such exported molecules are the protein components of cell walls (e.g., porins or lipoproteins) or the exocellular enzymes that are secreted by many bacteria into their culture medium, such as penicillinases, proteinases and amylases. Other proteins made within the cell may pass into the cytoplasmic membrane and remain there (e.g., enzymes such as cytochromes and membrane-bound dehydrogenases). The mechanism by which transport of these proteins occurs into or across the cytoplasmic membrane is unknown. A related question is: How does a cell "know" which of the many kinds of proteins within the cell to transport out of the cell? This question has been partially answered: The genes that code for these proteins carry a message that results in the addition of a sequence of about 20 extra amino acids (the signal peptide) to the proteins during their synthesis

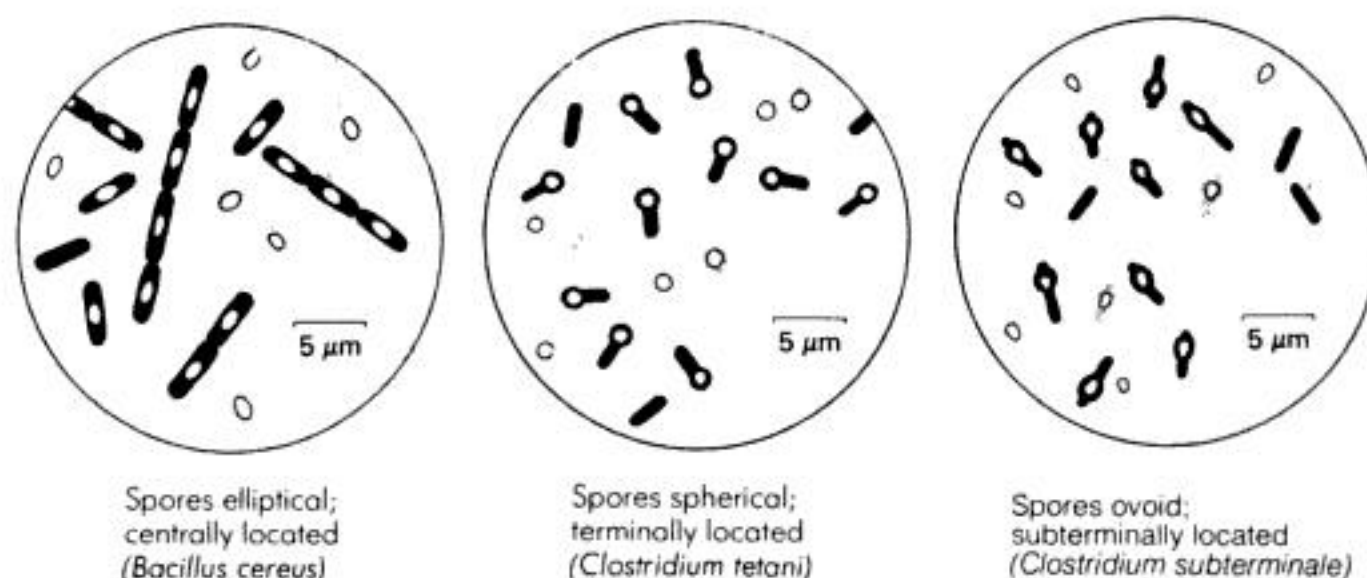
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**Figure 5-29.** Drawings showing the location, size, and shape of endospores in cells of various species of *Bacillus* and *Clostridium* (Erwin F. Lessel, illustrator.)



area near the center of the cell that is regarded as a nuclear structure, and the DNA of the cell is confined to this area. Because it is not a discrete nucleus, this nebulous structure has been designated by such terms as the *nucleoid*; the *chromatin body*; the *nuclear equivalent*; and even the *bacterial chromosome*, since it consists of a single, circular DNA molecule in which all the genes are linked. The nucleoid can be made visible under the light microscope by Feulgen staining, which is specific for DNA. By electron microscopy it appears as a light area with a delicate fibrillar structure (for example, see Figs. 5-22 and 5-26). The behavior of the nucleoid in growing, dividing bacteria has been observed by use of phase-contrast microscopy with a medium having a high refractive index.

## SPORES AND CYSTS

Certain species of bacteria produce spores, either within the cell (*endospores*) or external to the cell (*exospores*). The spore is a metabolically dormant form which, under appropriate conditions, can undergo germination and outgrowth to form a vegetative cell.

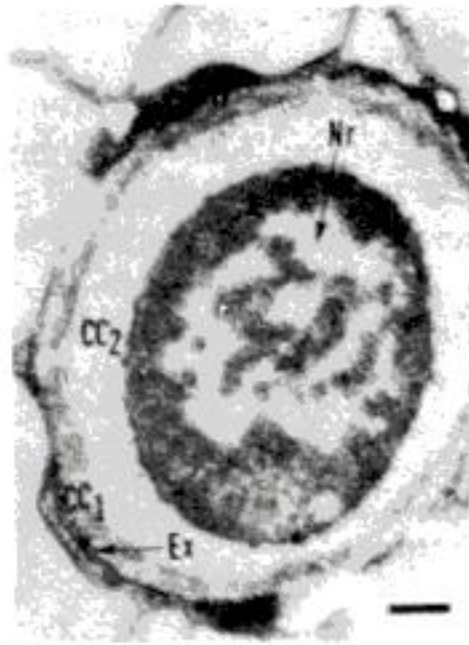
### Endospores

These structures are unique to bacteria. They are thick-walled, highly refractile bodies that are produced (one per cell) by *Bacillus*, *Clostridium*, *Sporosarcina*, *Thermoactinomyces*, and a few other genera. The shapes of endospores and also their location within the vegetative cell vary depending on the species (Fig. 5-29). The structural changes that occur during the development of endospores have been extensively studied in *Bacillus* and *Clostridium* species (Fig. 5-30). Endospores are usually produced by cells growing in rich media but which are approaching the end of active growth. Various factors such as aging or heat treatment are needed to activate the dormant spores (i.e., permit them to be able to undergo germination and outgrowth when they are placed in a suitable medium).

Endospores are extremely resistant to desiccation, staining, disinfecting chemicals, radiation, and heat. For example, the endospores of *Clostridium botulinum* type A have been reported to resist boiling for several hours. The degree of heat resistance of endospores varies with the bacterial species, but most can resist treatment at 80°C for at least 10 minutes. What causes this heat resistance has

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**Figure 5-33.** Fine structure of an *Azotobacter* cyst. The exosporium (Ex) and the two layers of exine (CC<sub>1</sub> and CC<sub>2</sub>) are visible. A nuclear region (Nr) and a cytoplasmic region containing ribosomes are observable within the central body. [Courtesy of Y.-T. Tchan and P. B. New, from N. R. Krieg (ed.), *Bergey's Manual of Systematic Bacteriology*, vol. 1, Williams & Wilkins, Baltimore, 1984.]

complex type produced by the genus *Azotobacter* (Fig. 5-33). Several other bacteria can differentiate into cystlike forms, but these seem to lack the degree of structural complexity characteristic of *Azotobacter* cysts.

## QUESTIONS

- 1 How does the cell's surface area/volume ratio compare with that of larger organisms? What advantages does a high surface area/volume ratio offer? What constraints does it place on a cell?
- 2 What bacterial cell structures may help to increase the cell's surface area/volume ratio?
- 3 If you performed a microscopic examination of an appropriately stained preparation of *Staphylococcus aureus*, would you expect all the cells to be arranged in clusters? Explain.
- 4 Explain why some species of cocci appear as chains but others appear in a cuboidal arrangement.
- 5 Draw a typical bacterial cell and identify all parts.
- 6 Contrast propulsion by a bacterial flagellum with that by a screw propeller on a submarine.
- 7 What functions might chemotaxis, phototaxis, and magnetotaxis have for bacteria in their natural habitats?
- 8 What problems associated with the shape and motility of spiroplasmas still remain to be solved?
- 9 What function might a capsule serve for the following bacteria?
  - (a) a pathogenic bacterium
  - (b) a soil bacterium where the soil is periodically subjected to drought conditions
  - (c) a bacterium living in a flowing stream
- 10 Why are Gram-negative eubacteria usually much easier to disrupt by sonic oscillation than Gram-positive eubacteria?
- 11 Compare the structure and chemistry of the cell walls of Gram-positive eubacteria versus those of Gram-negative eubacteria. List some major differences between the cell walls of archaeobacteria versus those of eubacteria.
- 12 What function do the porins of the outer membrane of a Gram-negative eubacterial cell wall serve? What functions do cytoplasmic membrane/outer membrane adhesions serve?



- 13 In what kinds of bacteria and in what kinds of bacterial cell structures would we be most likely to find the following compounds: (a) peptidoglycan, (b) teichoic acids, (c) calcium dipicolinate, (d) cholesterol, (e) lipopolysaccharide, (f) phytanols ether-linked to glycerol?
- 14 Is spore formation in bacteria a method of reproduction or a means of multiplication? Explain.
- 15 What are the similarities and differences between protoplasts and spheroplasts?
- 16 Is it proper to refer to bacterial cells as containing a typical nucleus? Explain.
- 17 Name several cytoplasmic inclusions or substances. What function might be associated with each of these?

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# Chapter 6

## The Cultivation of Bacteria

### OUTLINE Nutritional Requirements

#### Nutritional Types of Bacteria

Phototrophs • Chemotrophs • Autotrophs and Heterotrophs • Obligate Parasites

#### Bacteriological Media

Types of Media • Preparation of Media

#### Physical Conditions Required for Growth

Temperature • Gaseous Requirements • Acidity or Alkalinity (pH) • Miscellaneous Physical Requirements

#### Choice of Media and Conditions of Incubation

Except for certain ecological studies where bacterial populations are examined in their natural habitats, bacteria are usually cultivated and studied under laboratory conditions. Numerous **media** (singular, **medium**) have been developed for bacterial cultivation. Because the nutritional requirements of bacteria vary widely, there are great differences in the chemical compositions of the media used in the laboratory. Bacteria also exhibit wide differences with respect to the physical conditions favoring their growth, such as temperature, pH, and gaseous environment. The successful cultivation of bacteria requires an awareness of all of these factors.

### NUTRITIONAL REQUIREMENTS

All forms of life, from microorganisms to human beings, share certain nutritional requirements for growth and normal functioning. The following observations substantiate this statement and also illustrate the great diversity of nutritional types found among bacteria.

- 1 All organisms require a **source of energy**. Some rely on chemical compounds for their energy and are designated as **chemotrophs**. Others can utilize radiant energy (light) and are called **phototrophs**. Both chemotrophs and phototrophs exist among bacteria (see Table 6-1 for examples).
- 2 All organisms require a **source of electrons** for their metabolism. Some organisms can use reduced inorganic compounds as electron donors and are termed **lithotrophs** (some may be **chemolithotrophs**, others **photolithotrophs**). Other organisms use organic compounds as electron donors and are called **organotrophs** (some are **chemoorganotrophs**, others **photoorganotrophs**). Examples appear in Table 6-1.
- 3 All organisms require **carbon** in some form for use in synthesizing cell compo-

**Table 6-1.** Nutritional Characterization of Bacteria

Bacteria	Energy		Electron Donor		Carbon for Assimilation	
	Phototrophic	Chemotrophic	Lithotrophic	Organotrophic	Autotrophic	Heterotrophic
<i>Chromatium okenii</i>	+		+		+	
<i>Rhodospirillum rubrum</i> (anaerobic conditions)	+			+		+
(aerobic conditions)		+		+		+
<i>Nitrosomonas europaea</i>		+	+		+	
<i>Desulfovibrio desulfuricans</i>		+	+			+
<i>Pseudomonas pseudoflava</i> (H <sub>2</sub> supplied)		+	+		+	
(no H <sub>2</sub> supplied)		+		+		+
<i>Escherichia coli</i>		+		+		+

nents. All organisms require at least small amounts of CO<sub>2</sub>. However, some can use CO<sub>2</sub> as their major, or even sole, source of carbon; such organisms are termed autotrophs. Others require organic compounds as their carbon source and are termed heterotrophs (Table 6-1).

- 4 All organisms require nitrogen in some form for cell components. Bacteria are extremely versatile in this respect. Unlike eucaryotes, some bacteria can use atmospheric nitrogen. Others thrive on inorganic nitrogen compounds such as nitrates, nitrites, or ammonium salts, and still others derive nitrogen from organic compounds such as amino acids.
- 5 All organisms require oxygen, sulfur and phosphorus for cell components. Oxygen is provided in various forms, such as water; component atoms of various nutrients; or molecular oxygen. Sulfur is needed for synthesis of certain amino acids (cysteine, cystine, and methionine). Some bacteria require organic sulfur compounds, some are capable of utilizing inorganic sulfur compounds, and some can even use elemental sulfur. Phosphorus, usually supplied in the form of phosphate, is an essential component of nucleotides, nucleic acids, phospholipids, teichoic acids, and other compounds.
- 6 All living organisms require metal ions, such as K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Fe<sup>2+</sup> for normal growth. Other metal ions are also needed but usually only at very low concentrations, such as Zn<sup>2+</sup>, Cu<sup>2+</sup>, Mn<sup>2+</sup>, Mo<sup>6+</sup>, Ni<sup>2+</sup>, B<sup>3+</sup>, and Co<sup>2+</sup>; these are often termed trace elements and often occur as contaminants of other components of culture media in amounts sufficient to support bacterial growth.

Not all the biological functions of metal ions are known, but Fe<sup>2+</sup>, Mg<sup>2+</sup>, Zn<sup>2+</sup>, Mo<sup>6+</sup>, Mn<sup>2+</sup>, and Cu<sup>2+</sup>, are known to be cofactors for various enzymes (see Chap. 9).

Most bacteria do not require Na<sup>+</sup>, but certain marine bacteria, cyanobacteria, and photosynthetic bacteria do require it. For those members of the archaeobacteria known as the "red extreme halophiles," the requirement is astonishing: they cannot grow with less than 12 to 15 percent NaCl! They require this high level of NaCl for maintenance of the integrity of their cell walls and for the stability and activity of certain of their enzymes.

- 7 All living organisms contain vitamins and vitaminlike compounds. These function either as coenzymes for several enzymes (see Chap. 9) or as the building blocks for coenzymes. Some bacteria are capable of synthesizing their entire requirement of vitamins from other compounds in the culture medium, but others



cannot do so and will not grow unless the required vitamins are supplied pre-formed to them in the medium (see Table 6-2). Research in bacterial nutrition led to the discovery of some of the vitamins required by humans, and metabolic studies with bacteria contributed to our understanding of how these vitamins are synthesized and how they function.

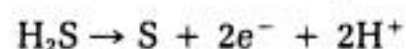
- 8 All living organisms require **water**, and in the case of bacteria all nutrients must be in aqueous solution before they can enter the cells. Water is a highly polar compound that is unequalled in its ability to dissolve or disperse cellular components and to provide a suitable milieu for the various metabolic reactions of a cell. Moreover, the high specific heat of water provides resistance to sudden, transient temperature changes in the environment. Water is also a chemical reactant, being required for the many hydrolytic reactions carried out by a cell.

## NUTRITIONAL TYPES OF BACTERIA

### Phototrophs

From the generalizations in the preceding paragraphs, it is apparent that bacteria can be divided into many groups on the basis of their nutritional requirements. The major separation is into two groups, phototrophs and chemotrophs.

Among the phototrophic bacteria are species that use inorganic compounds as their source of electrons (i.e., photolithotrophs). For example, *Chromatium okenii* uses  $H_2S$  as its electron donor, oxidizing it to elemental sulfur:



Some phototrophic bacteria use organic compounds such as fatty acids and alcohols as electron donors and are therefore photoorganotrophs. For example, *Rhodospirillum rubrum* can use succinate as an electron donor:



Certain phototrophic bacteria are not restricted to being phototrophic. As indicated before, chemotrophs rely on chemical compounds rather than light for their energy, and under some circumstances a phototrophic bacterium can grow as a chemotroph. For example, in the absence of  $O_2$  (i.e., under anaerobic conditions) *R. rubrum* is dependent on light as its source of energy and lives as a photoorganotroph; however, in the presence of  $O_2$  it can grow in the dark as a chemoorganotroph.

### Chemotrophs

Among the chemotrophic bacteria are species that use inorganic compounds as

**Table 6-2.** Vitamin Requirements for Some Bacteria

Vitamin	Species Exhibiting Requirement (or Growth Stimulation)
Thiamine ( $B_1$ )	<i>Bacillus anthracis</i>
Riboflavin	<i>Clostridium tetani</i>
Niacin (nicotinic acid)	<i>Brucella abortus</i>
Pyridoxine ( $B_6$ )	<i>Lactobacillus</i> spp.
Biotin	<i>Leuconostoc mesenteroides</i>
Pantothenic acid	<i>Morganella morganii</i>
Folic acid	<i>Leuconostoc dextranicum</i>
Cobalamin ( $B_{12}$ )	<i>Lactobacillus</i> spp.
Vitamin K	<i>Bacteroides melaninogenicus</i>



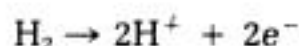
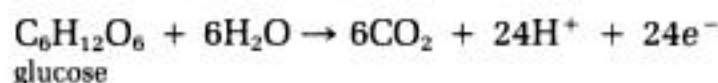
their source of electrons (i.e., chemolithotrophs). For example, bacteria of the genus *Nitrosomonas* use ammonia as their electron source, obtaining energy by oxidizing ammonia to nitrite:



This reaction involves a net transfer of 6 electrons, causing a valence change of the nitrogen atom from  $-3$  to  $+3$ .

Many other chemotrophic bacteria use organic compounds, such as sugars and amino acids, as electron donors and are therefore chemoorganotrophs.

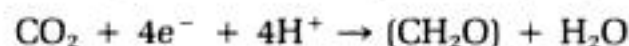
Certain bacteria can grow as either chemolithotrophs or chemoorganotrophs. For example, *Pseudomonas pseudoflava* can use either the organic compound glucose or the inorganic compound  $\text{H}_2$  as its source of electrons:



## Autotrophs and Heterotrophs

### Autotrophs

As indicated before, the chemolithotrophic bacteria of the genus *Nitrosomonas* are able to oxidize ammonia to nitrite, thereby obtaining sufficient energy to assimilate the carbon of  $\text{CO}_2$  into cell components ( $\text{CO}_2$  fixation):



where  $(\text{CH}_2\text{O})$  represents carbohydrate. Organisms that can use  $\text{CO}_2$  as their sole source of carbon for assimilation are termed autotrophs.

Until recently it was thought that all chemolithotrophic bacteria were autotrophs. Although this is true for most chemolithotrophs, a few are now recognized as being chemolithotrophic heterotrophs (**mixotrophs**); i.e., they obtain energy by utilizing inorganic electron donors, but obtain most of their carbon from organic compounds. One such organism is *Desulfovibrio desulfuricans*, which uses electrons from  $\text{H}_2$  for the reduction of sulfate, yet derives most of its carbon from organic compounds in the culture medium.

Some autotrophs are facultative autotrophs; i.e., they can either live as autotrophs, deriving their carbon from  $\text{CO}_2$ , or they can live as heterotrophs, deriving their carbon from organic compounds. For example, *P. pseudoflava* can live as a heterotroph, using glucose as a source of carbon for assimilation (and also as its source of electrons, as mentioned above); however, if  $\text{H}_2$  is provided as the electron source, then it can use  $\text{CO}_2$  as its sole carbon source and can grow as an autotroph.

**Cultivation of Autotrophs.** In terms of chemical complexity of nutrient substances required for growth, the autotrophic bacteria exhibit the simplest requirements. For example, a medium of the composition shown in Table 6-3 supports the growth of *Nitrosomonas europaea*. (Because this medium is composed of known chemical compounds, it is called a **chemically defined** or **synthetic medium**.) The fact that an organism can grow and reproduce in such a mixture of inorganic compounds indicates that it has an elaborate capacity for synthesis. That is, the organism can transform these compounds into the carbohydrates, proteins, nucleic acids, lipids, vitamins, and other complex organic substances that constitute the living cell.

**Table 6-3.** Medium for *Nitrosomonas europaea*

Ingredient	Amount
$\text{NH}_4\text{Cl}^*$	0.8 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g
$\text{K}_2\text{HPO}_4$	0.016 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.02 g
Chelated iron	0.001 g
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.0002 g
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.0001 g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.0001 g
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.00002 g
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.000002 g
Distilled water	1,000 ml
Atmospheric $\text{CO}_2$ †	

\* The ammonium salt serves not only as the nitrogen source for this organism but also as the electron donor. The organism obtains energy by oxidizing ammonium ions to nitrite ions.

† Carbon dioxide is the sole carbon source.



**Table 6-4.** Minimal Nutritional Requirements of Some Heterotrophic Bacteria

Bacteria	Inorganic Salts	Organic Carbon	Atmo-spheric N <sub>2</sub>	Inorganic Nitrogen	One Amino Acid	Two or More Amino Acids	One Vitamin	Two or More Vitamins
<i>Azospirillum brasilense</i>	+	+	+					
<i>Escherichia coli</i>	+	+		+				
<i>Salmonella typhi</i>	+	+		+	+			
<i>Proteus vulgaris</i>	+	+		+	+		+	
<i>Staphylococcus aureus</i>	+	+		+		+	+	
<i>Lactobacillus acidophilus</i>	+	+		+		+		+

## Heterotrophs

Heterotrophic bacteria have been studied more extensively than the autotrophs because heterotrophs, in a sense, are of more immediate concern to us: it is here that we find all the species that cause diseases of human beings, other animals, and plants, as well as those that constitute the greater part of the microbial population in our immediate environment. However, we need to emphasize this does not mean that autotrophs are less important. On the contrary, they are of utmost importance in less conspicuous but indispensable processes in nature such as the cycling of elements through biological systems.

**Cultivation of Heterotrophs.** The heterotrophic bacteria, although they constitute one major nutritional group, vary considerably in the specific nutrients required for growth, particularly with respect to their organic carbon sources, nitrogen sources, and vitamin requirements. As indicated in Table 6-4, the requirements may be relatively simple or complex, depending on the species. This is shown more specifically in Table 6-5, where chemically defined media for the growth of *Escherichia coli* and lactobacilli are compared. From this table it is evident that *E. coli* has much simpler nutritional requirements than lactobacilli. Organisms such as lactobacilli that have elaborate requirements for specific nutrients, i.e., vitamins and other growth-promoting substances, are designated **fastidious heterotrophs**.

## Obligate Parasites

Some bacteria have not yet been successfully cultivated on an artificial medium, and their nutritional and physical requirements are not understood. At present, such bacteria can be propagated only in association with a living host which, in a sense, serves as the medium. One example is the bacterium that causes leprosy, *Mycobacterium leprae*, which can be cultivated by infecting mice or armadillos. Other examples include the rickettsias, the chlamydias, and the spirochete that causes syphilis, *Treponema pallidum*.

## BACTERIOLOGICAL MEDIA

Chemically defined media are needed for the cultivation of autotrophs and are also useful for defining the nutritional requirements of heterotrophs. However, for the routine cultivation of heterotrophs, chemically defined media are not generally used. Instead, certain complex raw materials such as **peptones**, **meat**

**Table 6-5.** Composition of Media Supporting Growth of Lactobacilli and *Escherichia coli* (Heterotrophic Bacteria)

MEDIUM FOR CULTIVATION OF LACTOBACILLI*	
Casein hydrolysate	5 g
Glucose	10 g
Solution A	10 ml
Solution B	5 ml
L-Asparagine	250 ml
L-Tryptophan	50 mg
L-Cystine	100 mg
DL-Methionine	100 mg
Cysteine	100 mg
Ammonium citrate	2 g
Sodium acetate (anhydrous)	6 g
Adenine, guanine, xanthine, and uracil, each	10 mg
Riboflavin, thiamine, panththenate, and niacin, each	500 µg
Pyridoxamine	200 µg
Pyridoxal	100 µg
Pyridoxine	200 µg
Inositol and choline, each	10 µg
p-Aminobenzoic acid	200 µg
Biotin	5 µg
Folic acid (synthetic)	3 µg
Make up to 1 liter with distilled water.	
Solution A: K <sub>2</sub> HPO <sub>4</sub> and KH <sub>2</sub> PO <sub>4</sub> , each 25 g, into distilled water to a volume of 250 ml	
Solution B: FeSO <sub>4</sub> · 7H <sub>2</sub> O, 0.5 g; MnSO <sub>4</sub> · 2H <sub>2</sub> O, 2.0 g; NaCl, 0.5 g; and MgSO <sub>4</sub> · 7H <sub>2</sub> O, 10g	
Dissolve in distilled water to a volume of 250 ml	
MEDIUM FOR CULTIVATION OF <i>E. COLI</i>	
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	1 g
Glucose	5 g
NaCl	5 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.2 g
K <sub>2</sub> HPO <sub>4</sub>	1 g
H <sub>2</sub> O	1,000 ml

\* SOURCE: M. Rogosa et al., *J Bacteriol*, 54:13, 1947.

extract, and yeast extract are used, and the resulting media support the growth of a wide variety of heterotrophic bacteria. Agar is included as a nonnutritive solidifying agent when a solid medium is desired. A description of these raw materials is given in Table 6-6. Examples of relatively simple liquid and solid media that support the growth of many common heterotrophs are nutrient broth and nutrient agar (Table 6-7). The addition of yeast extract to each of these formulas improves the nutrient quality, since yeast extract contains several of the B vitamins and other growth-promoting substances. Other complex supplements such as bovine rumen fluid, animal blood, blood serum, or extracts of plant and animal tissues may be required for the cultivation of certain fastidious heterotrophs.

## Types of Media

Many special-purpose media are needed to facilitate recognition, enumeration, and isolation of certain types of bacteria. To meet these needs, the microbiologist



**Table 6-6.** Characteristics of Several Complex Materials Used as Ingredients of Media

Raw Material	Characteristic	Nutritional Value
Beef extract	An aqueous extract of lean beef tissue concentrated to a paste	Contains the water-soluble substances of animal tissue, which include carbohydrates, organic nitrogen compounds, water-soluble vitamins, and salts
Peptone	The product resulting from the digestion of proteinaceous materials, e.g., meat, casein, and gelatin; digestion of the protein material is accomplished with acids or enzymes; many different peptones (depending upon the protein used and the method of digestion) are available for use in bacteriological media; peptones differ in their ability to support growth of bacteria	Principal source of organic nitrogen; may also contain some vitamins and sometimes carbohydrates, depending upon the kind of proteinaceous material digested
Agar	A complex carbohydrate obtained from certain marine algae; processed to remove extraneous substances	Used as a solidification agent for media; agar, dissolved in aqueous solutions, gels when the temperature is reduced below 45°C; agar not considered a source of nutrient to the bacteria
Yeast extract	An aqueous extract of yeast cells, commercially available as a powder	A very rich source of the B vitamins; also contains organic nitrogen and carbon compounds

**Table 6-7.** Composition of Nutrient Broth and Nutrient Agar

Nutrient broth	
Beef extract	3 g
Peptone	5 g
Water	1,000 ml
Nutrient agar	
Beef extract	3 g
Peptone	5 g
Agar	15 g
Water	1,000 ml

has available numerous media which, on the basis of their application or function, may be classified as follows.

### Selective Media

These media provide nutrients that enhance the growth and predominance of a particular type of bacterium and do not enhance (and may even inhibit) other types of organisms that may be present. For instance, a medium in which cellulose is the only carbon source will specifically select for or enrich the growth of cellulose-utilizing organisms when it is inoculated with a soil sample containing many kinds of bacteria. As an example of a different type of selective medium, the isolation of the gonorrhea-causing organism, *Neisseria gonorrhoeae*, from a clinical specimen is facilitated by the use of media containing certain antibiotics; these antibiotics do not affect *N. gonorrhoeae* but do inhibit the growth of contaminating bacteria.

### Differential Media

Certain reagents or supplements, when incorporated into culture media, may allow differentiation of various kinds of bacteria. For example, if a mixture of bacteria is inoculated onto a blood-containing agar medium (blood agar), some of the bacteria may hemolyze (destroy) the red blood cells; others do not. Thus one can distinguish between hemolytic and nonhemolytic bacteria on the same medium.



**Assay Media**

Media of prescribed compositions are used for the assay of vitamins, amino acids, and antibiotics. Media of special composition are also available for testing disinfectants.

**Media for Enumeration of Bacteria**

Specific kinds of media are used for determining the bacterial content of such materials as milk and water. Their composition must adhere to prescribed specifications.

**Media for Characterization of Bacteria**

A wide variety of media are conventionally used to determine the type of growth produced by bacteria, as well as to determine their ability to produce certain chemical changes.

**Maintenance Media**

Satisfactory maintenance of the viability and physiological characteristics of a culture over time may require a medium different from that which is optimum for growth. Prolific, rapid growth may also be associated with rapid death of the cells at the end of the growth phase. For example, glucose in a medium frequently enhances growth, but acid harmful to the cells is likely to be produced. Therefore, omission of the glucose is preferable in a maintenance medium.

**Solid and Semisolid Media**

In addition to liquid media, solid and semisolid media are widely used for cultivation of bacteria. Solid media are useful for isolating bacteria or for determining the characteristics of colonies. The solidifying agent is usually agar, which at concentrations of 1.5 to 2.0 percent forms firm, transparent gels that are not degraded by most bacteria. Silica gel is sometimes used as an inorganic solidifying agent for autotrophic bacteria.

Semisolid media, prepared with agar at concentrations of 0.5 percent or less, have a soft, custardlike consistency and are useful for the cultivation of microaerophilic bacteria (see Gaseous Requirements later in this chapter) or for determination of bacterial motility.

**Preparation of Media**

Some naturally occurring substances are used for the cultivation of bacteria. Notable among these is milk, usually skimmed rather than whole. Such natural materials are merely dispensed into tubes or flasks and sterilized before use. Media of the nutrient broth or nutrient agar type are prepared by compounding the required individual ingredients or, more conveniently, by adding water to a dehydrated product which contains all the ingredients. Practically all media are available commercially in powdered form.

The preparation of bacteriological media usually involves the following steps:

- 1 Each ingredient, or the complete dehydrated medium, is dissolved in the appropriate volume of distilled water.
- 2 The pH of the fluid medium is determined with a pH meter and adjusted if necessary.
- 3 If a solid medium is desired, agar is added and the medium is boiled to dissolve the agar.
- 4 The medium is dispensed into tubes or flasks.
- 5 The medium is sterilized, generally by autoclaving. Some media (or specific ingredients) that are heat-labile are sterilized by filtration.



## PHYSICAL CONDITIONS REQUIRED FOR GROWTH

### Temperature

In addition to knowing the proper nutrients for the cultivation of bacteria, it is also necessary to know the physical environment in which the organisms will grow best. Just as bacteria vary greatly in their nutritional requirements, so do they exhibit diverse responses to physical conditions such as temperature, gaseous conditions, and pH.

Since all processes of growth are dependent on chemical reactions and since the rates of these reactions are influenced by temperature, the pattern of bacterial growth can be profoundly influenced by this condition. The temperature that allows for most rapid growth during a short period of time (12 to 24 h) is known as the **optimum growth temperature**. (It should be noted, however, that the optimum growth temperature thus defined may not necessarily be optimum for other cellular activities.)

Table 6-8 shows the optimum temperature for several bacteria and also the range of temperatures within which they will grow. It can be seen that the maximum temperature at which growth occurs is usually quite close to the optimum temperature, whereas the minimum temperature for growth is usually much lower than the optimum. On the basis of their temperature relationships, bacteria are divided into three main groups:

- 1 **Psychrophiles** are able to grow at 0°C or lower, though they grow best at higher temperatures. Many microbiologists restrict the term *psychrophile* to organisms that can grow at 0°C but have an optimum temperature of 15°C or lower and a maximum temperature of about 20°C; the term *psychrotroph* or *facultative psychrophile* is used for those organisms able to grow at 0°C but which grow best at temperatures in the range of about 20 to 30°C (e.g., see Fig. 6-1).

During isolation of strict psychrophiles it is usually necessary to maintain the source samples (for example, Antarctic soil samples) at cold temperatures from the time they are collected and also to chill all media before attempting isolation. This is because strict psychrophiles usually die if they are even temporarily exposed to room temperature. Even at optimum growth temperatures, it often takes two or three weeks for colonies of psychrophiles to develop.

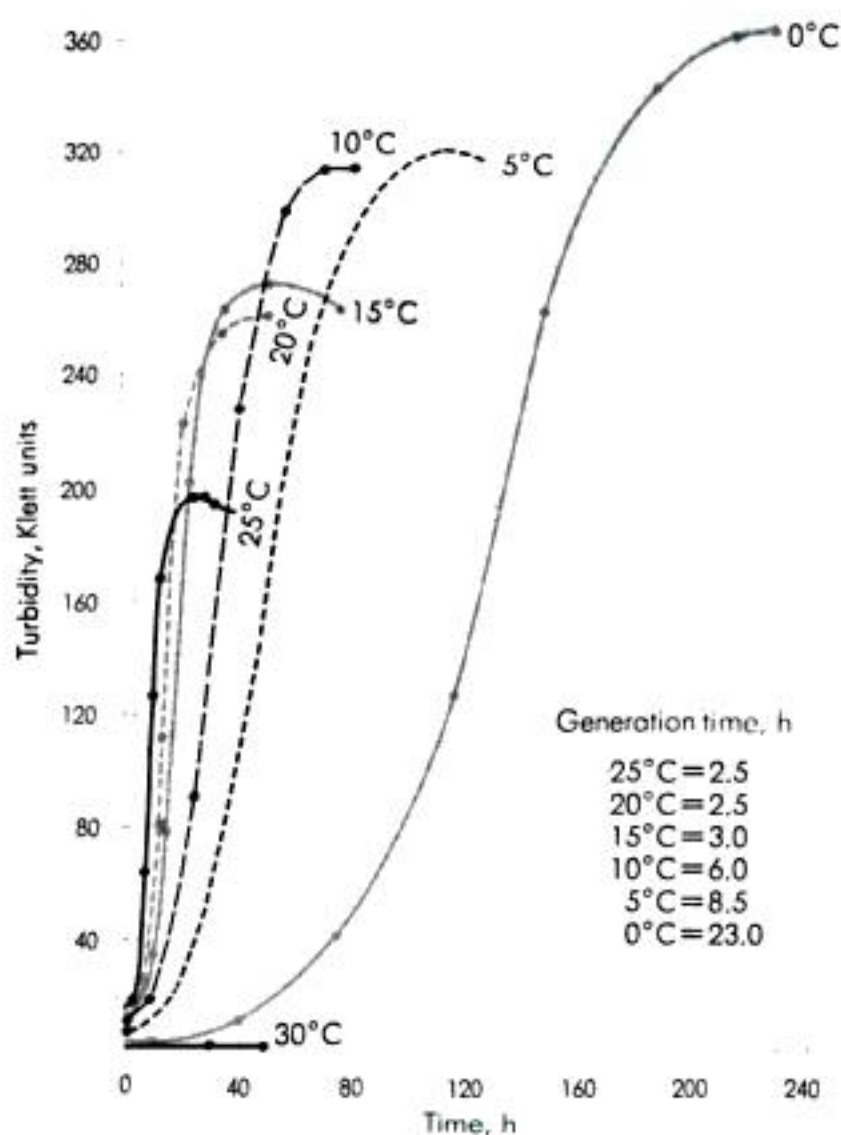
The physiological factors responsible for the low temperature maxima for strict psychrophiles are not entirely clear, but some factors that have been implicated are heat instability of ribosomes and various enzymes, increased leakage of cell

**Table 6-8.** Characteristics of Several Species of Bacteria with Regard to Temperatures at Which They Grow

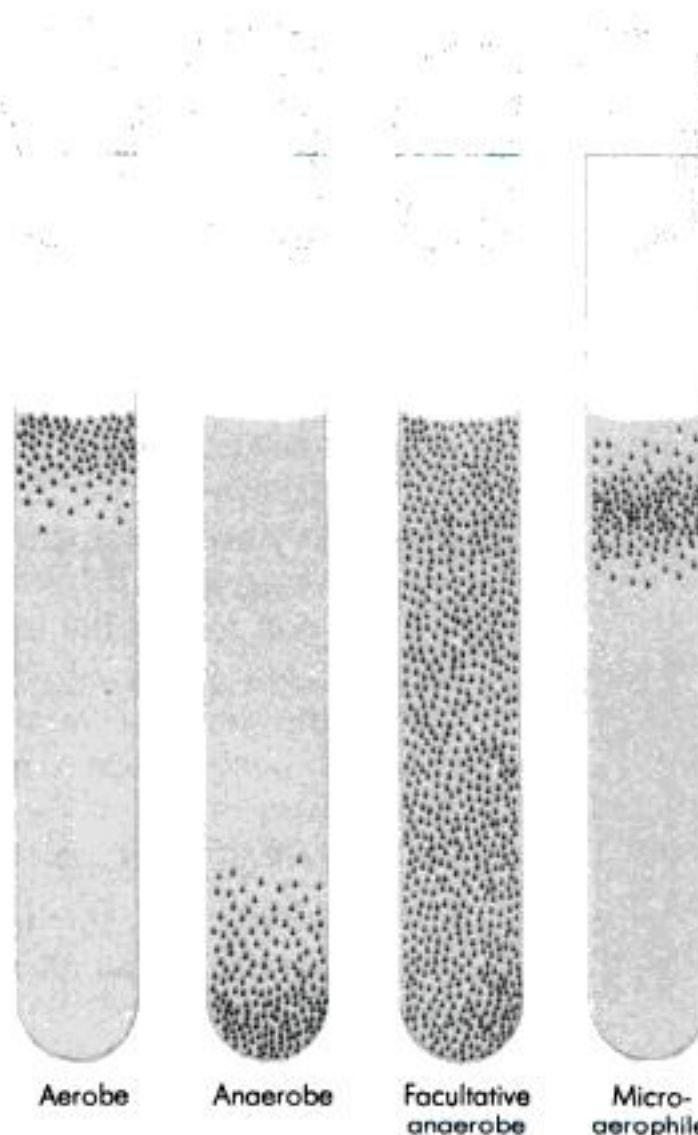
	Temperature of Growth, °C		
	Minimum	Optimum	Maximum
<i>Vibrio marinus</i> strain MP-1	-1	15	20
<i>Vibrio psychroerythrus</i>	0	15	19
<i>Pseudomonas fluorescens</i>	4	25-30	40
<i>Staphylococcus aureus</i>	6.5	30-37	46
<i>Corynebacterium diphtheriae</i>	15	37	40
<i>Neisseria gonorrhoeae</i>	30	35-36	38.5
<i>Streptococcus thermophilus</i>	20	40-45	50
<i>Thermoactinomyces vulgaris</i>	27-30	60	65-70
<i>Thermus aquaticus</i>	40	70-72	79

SOURCE: Data from R. Y. Morita, *Bacteriol Rev*, 39:144, 1975, and from *Bergey's Manual of Determinative Bacteriology*, 8th ed, Williams & Wilkins, Baltimore, 1974.





**Figure 6-1.** Effect of temperature on the growth of a psychrotrophic *Bacillus* sp. Note that rate of growth (measured turbidimetrically in Klett units) is more rapid at 25°C than at 0°C, although the total quantity of cells at the termination of growth is greater at the lower temperature. (Courtesy of J. L. Stokes in *Low Temperature Biology of Food Stuffs*, Pergamon, New York, 1968.)



**Figure 6-2.** Schematic illustration of the growth of bacteria in deep agar tubes, showing differences in response to atmospheric oxygen.

components, and impaired transport of nutrients above the maximum temperature.

- 2 **Mesophiles** grow best within a temperature range of approximately 25 to 40°C. For example, all bacteria that are pathogenic for humans and warm-blooded animals are mesophiles, most growing best at about body temperature (37°C).
- 3 **Thermophiles** grow best at temperatures above 45°C. The growth range of many thermophiles extends into the mesophilic region; these species are designated **facultative thermophiles**. Other thermophiles cannot grow in the mesophilic range; these are termed **true thermophiles**, **obligate thermophiles**, or **stenothermophiles**.

Factors that have been implicated in the ability to grow at high temperatures are an increased thermal stability of ribosomes, membranes, and various enzymes. Loss of the fluidity that exists within the lipid bilayer of the cytoplasmic membrane may be a factor governing the minimum temperature.

It is important to note that a bacterial species may not manifest the same characteristics in every detail when grown at different temperatures. For example, *Serratia marcescens* forms a blood-red to orange pigment when cultured at 25°C but produces little or no pigment when cultured at 37°C. Similarly, *Lactobacillus plantarum* does not require the amino acid phenylalanine for growth when cultured at 25°C but does require it at 37°C.

## Gaseous Requirements

The principal gases that affect bacterial growth are oxygen and carbon dioxide. Bacteria display such a wide variety of responses to free oxygen that it is convenient to divide them into four groups on the following bases:

- 1 **Aerobic bacteria** require oxygen for growth and can grow when incubated in an air atmosphere (i.e., 21 percent oxygen).
- 2 **Anaerobic bacteria** do not use oxygen to obtain energy; moreover, oxygen is toxic for them and they cannot grow when incubated in an air atmosphere. Some can tolerate low levels of oxygen (nonstringent or tolerant anaerobes), but others (stringent or strict anaerobes) cannot tolerate even low levels and may die upon brief exposure to air.
- 3 **Facultatively anaerobic bacteria** do not require oxygen for growth, although they may use it for energy production if it is available. They are not inhibited by oxygen and usually grow as well under an air atmosphere as they do in the absence of oxygen.
- 4 **Microaerophilic bacteria** require low levels of oxygen for growth but cannot tolerate the level of oxygen present in an air atmosphere.

Figure 6-2 shows diagrammatically how these four classes can be distinguished by their patterns of growth in tubes in deep agar media where the diffusion of oxygen into the medium is a controlling factor.

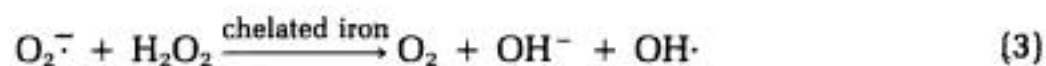
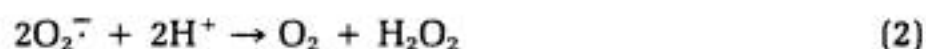
## Oxygen Toxicity

Oxygen is both beneficial and poisonous to living organisms. It is beneficial because its strong oxidizing ability makes it an excellent terminal electron acceptor for the energy-yielding process known as respiration. However, oxygen is also a toxic substance. Aerobic and facultative organisms have developed protective mechanisms that greatly mitigate this toxicity, but microaerophiles and anaerobes are deficient in these mechanisms and are restricted to habitats where little or no oxygen is present. The following factors are among those that have been implicated in oxygen toxicity.

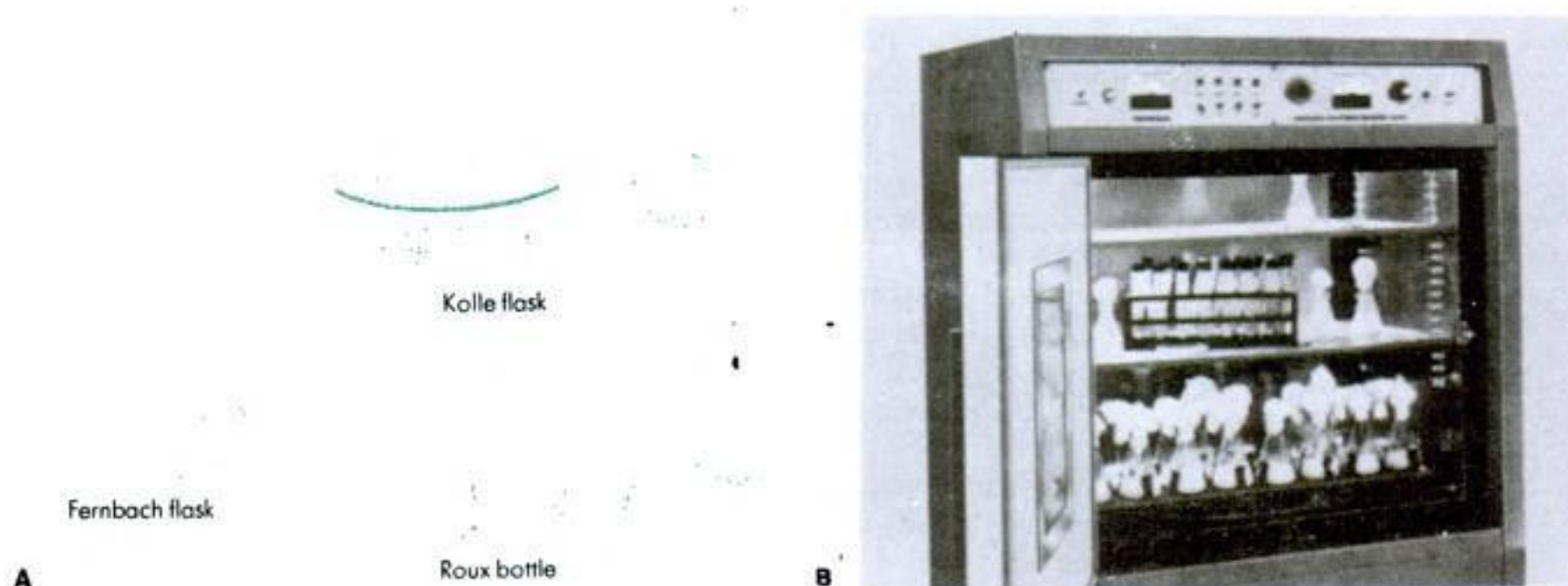
- 1 **Oxygen inactivation of enzymes.** Molecular oxygen can directly oxidize certain essential reduced groups, such as thiol (-SH) groups, or enzymes, resulting in enzyme inactivation. For instance, the enzyme complex known as nitrogenase, responsible for nitrogen fixation, is irreversibly destroyed by even small amounts of oxygen.
- 2 **Damage due to toxic derivatives of oxygen.** Various cellular enzymes catalyze chemical reactions involving molecular oxygen; some of these reactions can result in addition of a single electron to an oxygen molecule, thereby forming a superoxide radical ( $O_2^{\cdot-}$ ):



Superoxide radicals can inactivate vital cell components. However, recent studies suggest that their greatest detrimental action is through production of even more toxic substances such as hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals ( $OH\cdot$ ) by means of the following reactions:



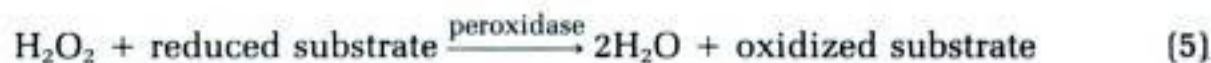




**Figure 6-3.** Methods for providing increased aeration during incubation. (A) Culture vessels of several designs that provide a large surface area for a shallow layer of medium. (B) An example of an incubator-shaker. The environmental chamber provides controlled conditions of temperature, humidity, and illumination. Within the chamber, flasks are fixed firmly on a platform which rotates in a circular manner, thus agitating the fluid medium constantly during incubation and exposing more culture surface to the gas phase. (Courtesy of New Brunswick Scientific Company.)

Hydroxyl radicals are among the most reactive free radicals known to organic chemistry and can damage almost every kind of molecule found in living cells. Hydrogen peroxide is not a free radical, but it is a powerful oxidizing agent that is highly toxic to many kinds of cells. Another toxic derivative of oxygen is an energized form known as **singlet oxygen**, ( $^1\Delta_g$ )O<sub>2</sub>, which is produced in biological systems by certain photochemical reactions.

Aerobic and facultative organisms have developed various protective mechanisms against the toxic forms of oxygen. One is the enzyme known as **superoxide dismutase**, which eliminates superoxide radicals by greatly increasing the rate of reaction 2 above. The hydrogen peroxide produced by this reaction can in turn be dissipated by **catalase** and **peroxidase** enzymes:



Note that elimination of either superoxide radicals or hydrogen peroxide can prevent the formation of the highly dangerous hydroxyl radicals, since both reactants are required for reaction (3).

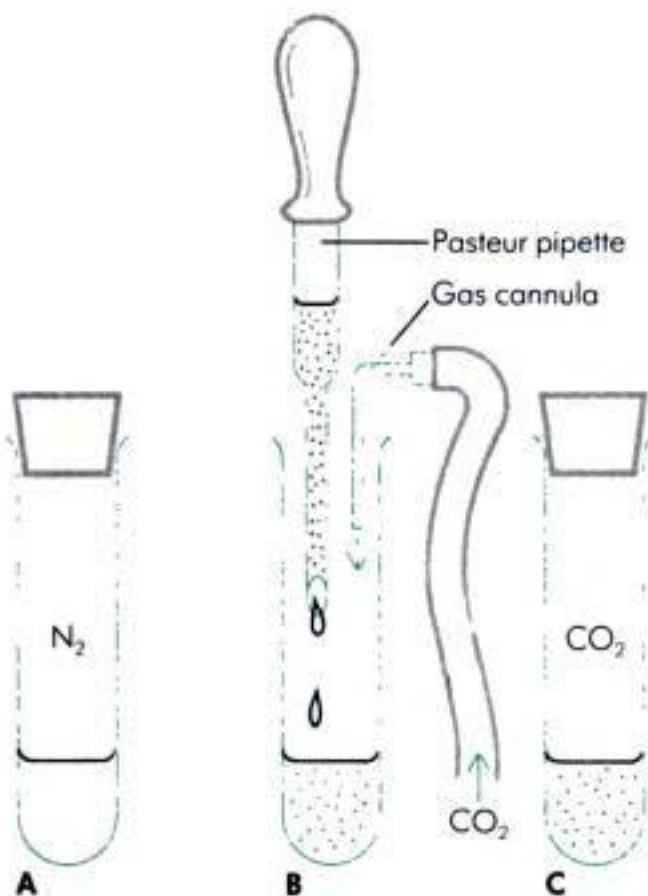
In general, anaerobic bacteria have either no superoxide dismutase or only relatively low levels compared to aerobes. Many anaerobes are also deficient in catalase and/or peroxidase. This may help to explain, at least in part, their sensitivity to oxygen, although other factors are probably involved as well.

**Cultivation of Aerobic Bacteria.** To grow aerobic or facultative bacteria in tubes or small flasks, incubation of the medium under normal atmospheric conditions is generally satisfactory. However, when aerobic organisms are to be grown in large quantities, it is advantageous to increase the exposure of the medium to the atmosphere. This can be accomplished by dispensing the medium in shallow layers, for which special containers are available. Aeration can also be increased by constantly shaking the inoculated liquid cultures (Fig. 6-3).



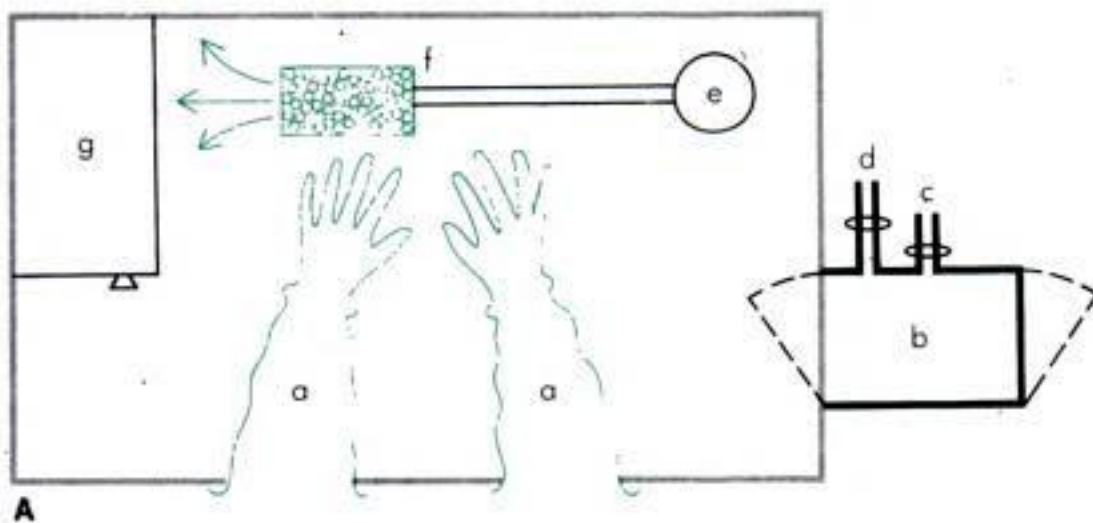
**Cultivation of Anaerobic Bacteria.** Stringent anaerobes can be grown only by taking special precautions to exclude all atmospheric oxygen from the medium. Such an environment can be established by using one of the following methods:

- 1 **Prereduced media.** During preparation, the culture medium is boiled for several minutes to drive off most of the dissolved oxygen. A reducing agent, e.g., cysteine, is added to further lower the oxygen content. Oxygen-free  $N_2$  is bubbled through the medium to keep it anaerobic. The medium is then dispensed into tubes which are being flushed with oxygen-free  $N_2$ , stoppered tightly, and sterilized by autoclaving. Such tubes can be stored for many months before being used. During inoculation, the tubes are continuously flushed with oxygen-free  $CO_2$  by means of a cannula (Fig. 6-4), restoppered, and incubated.
- 2 **Anaerobic chamber.** This refers to a plastic anaerobic glove box (Fig. 6-5) that



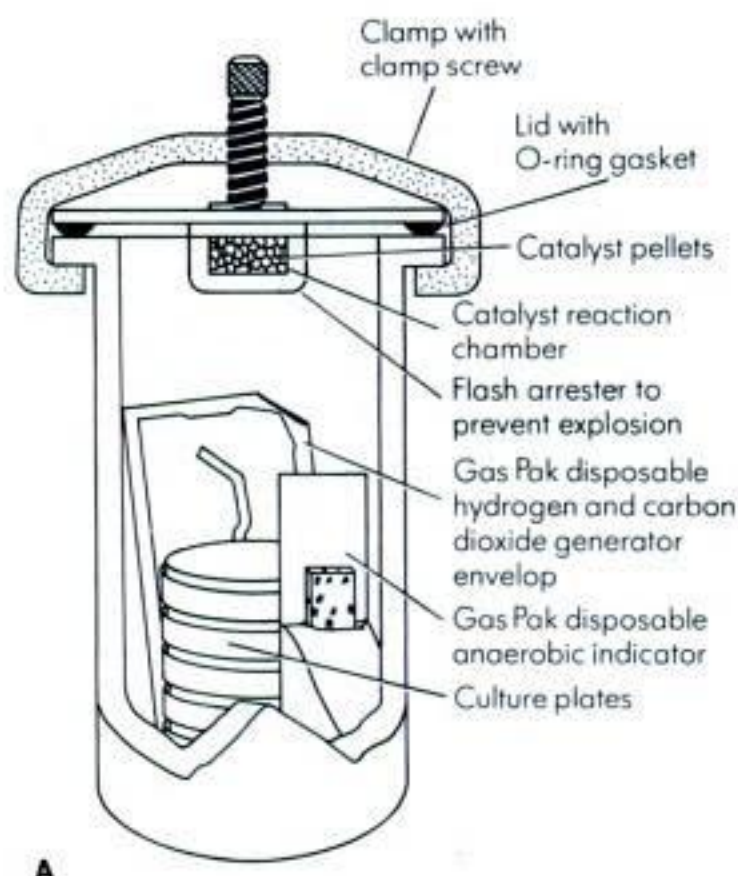
**Figure 6-4.** Use of prereduced media for cultivation of stringent anaerobes. (A) Tube of prereduced medium containing an atmosphere of oxygen-free  $N_2$ . (B) To inoculate, the stopper is removed and a gas cannula inserted to flush the tube continuously with oxygen-free  $CO_2$  and maintain anaerobic conditions. The medium is inoculated with a few drops of culture by means of a Pasteur pipette. (C) After inoculation the tube is restoppered and incubated.

**Figure 6-5.** (A) Schematic diagram of the various parts of an anaerobic chamber (top view). (a) Glove ports and rubber gloves that allow the operator to perform manipulations within the chamber. (b) Air lock with inner and outer doors. Media are placed within the air lock with the inner door remaining sealed; air is removed by a vacuum pump connection (c) and replaced with  $N_2$  through (d). The inner door is opened and the media are placed within the main chamber, which contains an atmosphere of  $H_2 + CO_2 + N_2$ . A circulator (e) circulates the gas atmosphere through pellets of palladium catalyst (f), causing any residual oxygen in the media to be used up by reaction with  $H_2$ . After media have become completely anaerobic they can be inoculated and placed in an incubator (g) located within the chamber. (B) Photograph of an anaerobic chamber. (Courtesy of The Germfree Laboratories, Inc.)





**Figure 6-6.** Anaerobic jar: GasPak system. (A) Media are inoculated and then placed in the jar. Water is added to the GasPak generator envelope, causing the evolution of  $H_2$  and  $CO_2$ . The  $H_2$  reacts with  $O_2$  on the surface of the palladium catalyst, forming water and establishing anaerobic conditions. The  $CO_2$  aids growth of fastidious anaerobes which sometimes fail to grow, or grow only poorly, in its absence. An anaerobic indicator strip (a pad saturated with methylene blue solution) changes from blue to colorless in the absence of oxygen. (B) The GasPak Anaerobic System with inoculated Petri dishes, the GasPak generator envelope, and the anaerobic indicator strip. (Courtesy of BBL Microbiology Systems.)



contains an atmosphere of  $H_2$ ,  $CO_2$ , and  $N_2$ . Culture media are placed within the chamber by means of an air lock which can be evacuated and refilled with  $N_2$ . From the air lock the media are placed within the main chamber. Any  $O_2$  in the media is slowly removed by reaction with the  $H_2$ , forming water; this reaction is aided by a palladium catalyst. After being rendered oxygen-free, the media are inoculated within the chamber (by means of the glove ports) and incubated (also within the chamber).

Nonstringent anaerobes can be cultured within an **anaerobic jar** such as that depicted in Fig. 6-6. Inoculated media are placed in the jar along with an  $H_2 + CO_2$  generating system. After the jar is sealed, the oxygen present in the atmosphere within the jar, as well as that dissolved in the culture medium, is gradually used up through reaction with the hydrogen in the presence of a catalyst.

### Acidity or Alkalinity (pH)

For most bacteria the optimum pH for growth lies between 6.5 and 7.5, and the limits generally lie somewhere between 5 and 9. However, a few bacteria prefer more extreme pH values for growth. For example, *Thiobacillus thiooxidans* has an optimum pH of 2.0 to 3.5 and can grow in a range between pH 0.5 and 6.0. On the other hand, an unclassified bacterium isolated from an alkaline spring in California was found to grow best at a pH of 9.0 to 9.5 and could grow within a range from 8.0 to 11.4.

When bacteria are cultivated in a medium originally adjusted to a given pH, for example, 7.0, it is very likely that this pH will change as a result of the chemical activities of the organism. If a carbohydrate is present it may be fermented or oxidized to organic acids, thus decreasing the pH of the medium. If the salt of an organic acid is supplied as a carbon source (e.g., sodium malate), its oxidation by bacteria will cause an increase in pH. Such shifts in pH may be so great that further growth of the organism is eventually inhibited.

*image  
not  
available*



- (c) The stenothermophile *Clostridium thermosaccharolyticum* from a can of spoiled corn
- (d) an extreme halophile from a sample of sea salt
- (e) a nitrogen-fixing bacterium from a soil sample
- 5 Most medically important anaerobic bacteria are nonstringent anaerobes, but some are the stringent type. Describe two methods recommended for cultivating the latter organisms.
- 6 Is nutrient broth a “universal” medium (that is, can it support the growth of every kind of bacteria)? Explain.
- 7 If you wanted to buffer a culture medium to keep the pH at 5.5, you would try to use a buffer having what  $pK_a$ ? Why?
- 8 List some of the ways whereby you could enhance the growth of an aerobic bacterium.
- 9 Under what circumstances might you wish to use silica gel as a solidifying agent for a culture medium?
- 10 Indicate the various toxic derivatives of oxygen and explain how aerobic organisms might protect themselves against these derivatives.

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- Krieg, N. R. (ed.): *Bergey’s Manual of Systematic Bacteriology*, vol. 1, Williams & Wilkins, Baltimore, 1984. This together with subsequent volumes provides detailed descriptions of the genera and species of bacteria, including the nutritional and physical conditions required for isolation and cultivation.
- Starr, M. P., H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (eds.): *The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria*, Springer-Verlag, New York, 1981. The introductory chapters of this book contain summaries of the various nutritional and physical factors that affect bacteria; other chapters provide specific information about the habitat, isolation, and cultivation of each bacterial group.



# Chapter 7

# Reproduction and Growth

## OUTLINE    **Reproduction**

Modes of Cell Division • New Cell Formation (Macromolecular Synthesis)

## **Growth**

Normal Growth Cycle (Growth Curve) of Bacteria • Transitional Periods Between Growth Phases • Synchronous Growth • Continuous Culture

## **Quantitative Measurement of Bacterial Growth**

Direct Microscopic Count • Electronic Enumeration of Cell Numbers • The Plate-Count Method • Membrane-Filter Count • Turbidimetric Methods • Determination of Nitrogen Content • Determination of the Dry Weight of Cells • Measurement of a Specific Chemical Change Produced on a Constituent of the Medium • The Relation of Turbidity Measurements to Direct Expressions of Growth • The Selection of a Procedure to Measure Growth • Importance of Quantitative Measurement of Growth

When bacteria are inoculated into a suitable medium and incubated under appropriate conditions, a tremendous increase in the number of cells occurs within a relatively short time. With some species the maximum population is reached within 24 h, but others require a much longer period of incubation to reach maximum growth. The term *growth* as commonly applied to bacteria and other microorganisms usually refers to *changes in the total population* rather than an increase in the size or mass of an individual organism. More frequently than not, the inoculum contains thousands of organisms; growth denotes the increase in number beyond that present in the original inoculum. Therefore, determination of growth requires quantitative measurement of the total population of cells or cell crops at the time of inoculation and again after incubation. In this chapter we will discuss how bacteria reproduce and how their growth can be measured.

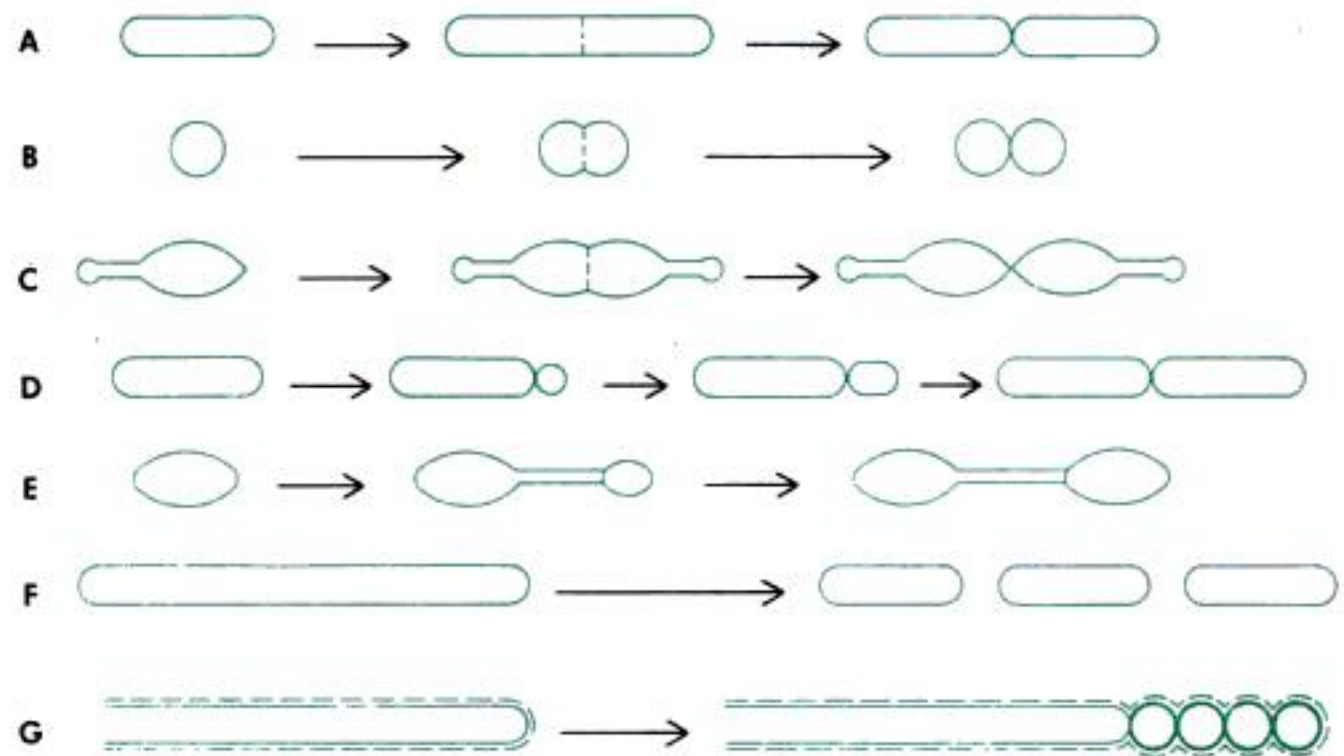
## **REPRODUCTION**

### **Modes of Cell Division**

#### **Binary Fission**

The most common, and no doubt the most important, mode of cell division in the usual growth cycle of bacterial populations is **transverse binary fission**, in which a single cell divides after developing a transverse septum (crosswall) (Fig. 7-1 A, B, C). Transverse binary fission is an asexual reproductive process. (Infrequently, in some species, binary fission may be preceded by a mating or conjugation of cells; this sexual process is discussed in Chap. 12.)





**Figure 7-1.** Schematic drawing of modes of cell division in various bacteria. Transverse binary fission occurs in *Bacillus subtilis* (A), *Streptococcus faecalis* (B), and the prosthecae bacterium *Prostheco bacterium fusiformis* (C); in the latter species the small round area at the tip of each prostheca is a holdfast—a means of attachment to surfaces. Budding occurs in *Rhodopseudomonas acidophila* (D) and *Hyphomicrobium vulgare* (E); in the latter species the mother cell produces a prostheca on which a terminal bud forms; this bud develops into a daughter cell. (F) Fragmentation occurs in the filamentous cells of a *Nocardia* species. (G) Formation of conidiospores by a *Streptomyces* species. A hypha that gives rise to spores is covered by a sheath (represented here by a dashed line); septation occurs at the hyphal tip to produce a chain of conidiospores still enclosed by the sheath.

## Budding

Some bacteria, such as *Rhodopseudomonas acidophila*, reproduce by **budding**, a process in which a small protuberance (bud) develops at one end of the cell; this enlarges and eventually develops into a new cell which separates from the parent (Fig. 7-1D). In some budding bacteria, such as *Hyphomicrobium* species, the bud may develop at the end of a prostheca (Fig. 7-1E).

## Fragmentation

Bacteria that produce extensive filamentous growth, such as *Nocardia* species, reproduce by **fragmentation** of the filaments into small bacillary or coccoid cells, each of which gives rise to new growth (Fig. 7-1F).

## Formation of Conidiospores or Sporangiospores

Species of the genus *Streptomyces* and related bacteria produce many spores per organism by developing crosswalls (septation) at the hyphal tips; each spore gives rise to a new organism (Fig. 7-1G).

## New Cell Formation (Macromolecular Synthesis)

A bacterial cell inoculated into a fresh medium selectively takes up nutrients from its environment. Many biochemical syntheses then take place. The nutrients are converted into cell substance—RNA, DNA, proteins, enzymes, and

other macromolecules. Cell mass and cell size increase, and new cell wall building blocks are synthesized. Subsequently, the process of binary fission is initiated, ultimately resulting in the formation of two new cells.

### Septum Formation

In transverse binary fission, septum formation does not begin until the chromosome content of the cell has been doubled; i.e., cell division is triggered by completion of DNA replication (discussed in Chap. 11). The first step is an inward growth of the cytoplasmic membrane at the middle of the cell; a mesosome is usually attached to the cytoplasmic membrane at this location, particularly in Gram-positive cells, and may have a role in the synthesis of new membrane material. The next step is the inward growth of the cell wall to form a septum that ultimately splits to allow separation of the two daughter cells.

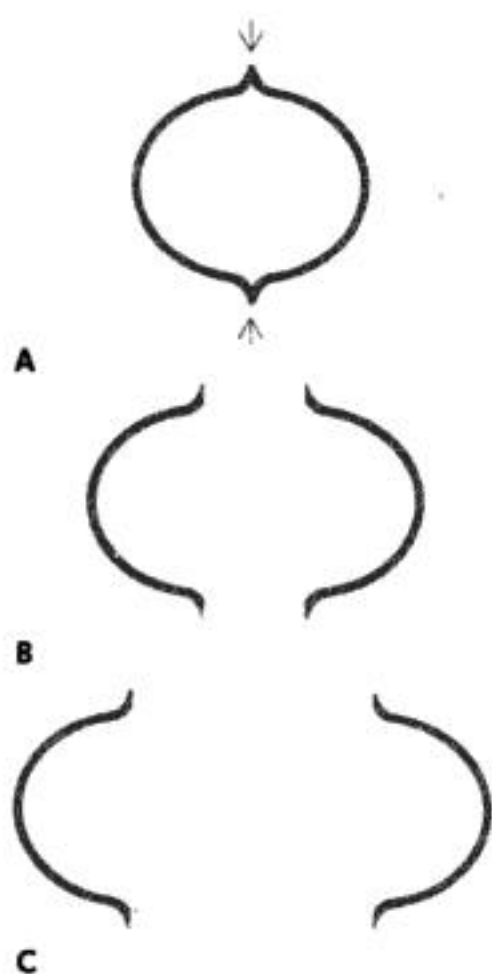
For example, during growth of the Gram-positive coccus *Streptococcus faecalis*, all of the new wall material formed by the dividing cell is made during synthesis of the septum. Septum formation begins beneath an equatorial ridge in the cell wall (see Figs. 7-2 and 5-26). New cell-wall material is synthesized in this region and, as the septum forms, this material becomes one half of the wall of each daughter cell. Some plasticity must be present in order for the new wall to achieve its final, more or less hemispherical shape; this is believed to be due to two factors: (1) the turgor pressure of the protoplast against the newly synthesized wall and (2) a certain amount of reorganization of the peptidoglycan due to breakage of some of the chemical bonds by hydrolytic enzymes and subsequent formation of new bonds at a different location.

In Gram-positive rods such as *Bacillus subtilis*, the transverse septum is formed in a manner similar to that for *S. faecalis*, although no ridge is present at the middle of the cell. Moreover, only about 15 percent of the new wall of a daughter cell is derived from formation of the septum. The remainder is synthesized along the cylindrical part of the cell, since a bacillus grows mainly by elongation rather than just by septum formation as does a coccus. Perhaps there are just a few discrete regions where new wall is synthesized and inserted into old wall, or perhaps new wall is made and inserted into old wall all along the length of the cell, but this is a question yet unanswered. However, there is strong evidence that the youngest portion of the cylindrical wall is that layer which is immediately adjacent to the cytoplasmic membrane. During growth and extension of the wall, the older, outer layers of the wall become more thinly spread out. They are eventually destroyed by degradative enzymes located in the cell wall and are replaced from below by the newer wall material. Thus the wall is not static like a plastic coating; rather, it is in a dynamic state, with old, outer material continually being destroyed and new, inner material continually being added as the cell elongates.

The stages of septum formation in Gram-negative bacilli such as *Escherichia coli* are depicted in Fig. 7-3.

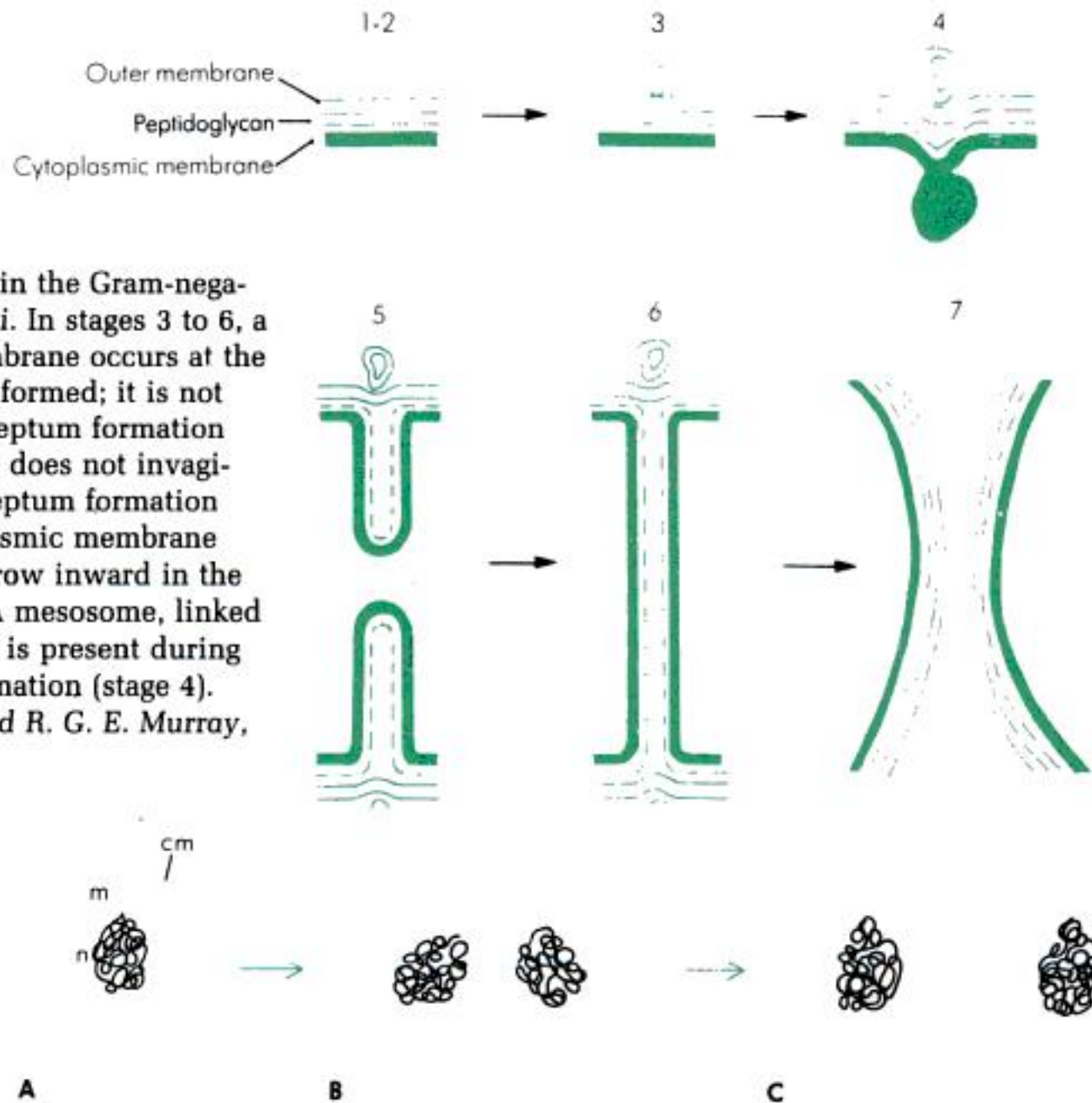
A number of basic questions remain to be answered concerning transverse binary fission:

- 1 What ensures that each daughter cell will receive a complete genome? That is, during septum formation in any bacterial cell, it is essential that the DNA be precisely distributed to the daughter cells so that each receives a complete genome.



**Figure 7-2.** Schematic diagram illustrating septum formation in Gram-positive cocci such as *Streptococcus faecalis*. (A) Synthesis of new wall begins at the equatorial ridge (arrows). (B) From this site new wall grows peripherally, pushing apart the hemispheres of old wall. Half of the entire wall of each daughter cell is derived from the septum.





**Figure 7-3.** Septum formation in the Gram-negative bacterium *Escherichia coli*. In stages 3 to 6, a bleb, or fold, of the outer membrane occurs at the site where the septum will be formed; it is not evident in the final stages of septum formation (stage 7). The outer membrane does not invaginate until the final stages of septum formation (stage 7); however, the cytoplasmic membrane and the peptidoglycan layer grow inward in the early stages (stages 4 and 5). A mesosome, linked to the cytoplasmic membrane, is present during the early stages of septum formation (stage 4). (Courtesy of I. D. J. Burdett and R. G. E. Murray, *J Bacteriol* 119:1039, 1974.)

**Figure 7-4.** Illustration of the hypothesized role of the central mesosome in segregation of DNA into daughter bacterial cells. (A) Cell before binary fission, showing cytoplasmic membrane (cm), central mesosome (m), and the nucleoid (n). (The cell wall is not shown.) In this model the nucleoid is shown attached to the mesosome. Evidence for such attachment has been obtained by electron microscopy of thin sections of bacteria. (B) During binary fission both the DNA of the nucleoid and its attachment site to the mesosome are duplicated. The mesosome begins to divide because of synthesis of membrane between the DNA-mesosome attachment sites. (C) During binary fission each mesosome is "pushed" to the middle of a daughter cell because of synthesis of cytoplasmic membrane between the mesosomes. Because a nucleoid is attached to each mesosome, the nucleoids become properly segregated into the daughter cells.

No mitotic apparatus for this segregation of daughter DNA molecules exists in bacteria; however, the cytoplasmic membrane, or the central mesosome derived from it, may play an equivalent role (Fig. 7-4).

- 2 What causes the septum to form at approximately the middle of the cell—why not near one of the poles? (Indeed, some mutants of *Escherichia coli* and *B. subtilis* have been obtained which often do form the septum near a pole, resulting



in a very small daughter cell (termed a **minicell**) which lacks DNA and therefore cannot multiply.)

### 3 How does the completion of DNA replication initiate septum formation?

It is apparent from these and other questions that although transverse binary fission may be a primitive means of reproduction compared to that which occurs in eucaryotes, it is by no means a simple process; rather, it is the result of a precisely orchestrated series of interdependent events, many of which are not yet completely understood.

## GROWTH

The most common means of bacterial reproduction is binary fission; one cell divides, producing two cells. Thus, if we start with a single bacterium, the increase in population is by geometric progression:

$$1 \rightarrow 2 \rightarrow 2^2 \rightarrow 2^3 \rightarrow 2^4 \rightarrow 2^5 \dots 2^n$$

where  $n$  = the number of generations. Each succeeding generation, assuming no cell death, doubles the population. The total population  $N$  at the end of a given time period would be expressed

$$N = 1 \times 2^n \quad (1)$$

However, under practical conditions, the number of bacteria  $N_0$  inoculated at time zero is not 1 but more likely several thousand, so the formula now becomes

$$N = N_0 \times 2^n \quad (2)$$

Solving Eq. (2) for  $n$ , we have

$$\begin{aligned} \log_{10} N &= \log_{10} N_0 + n \log_{10} 2 \\ n &= \frac{\log_{10} N - \log_{10} N_0}{\log_{10} 2} \end{aligned} \quad (3)$$

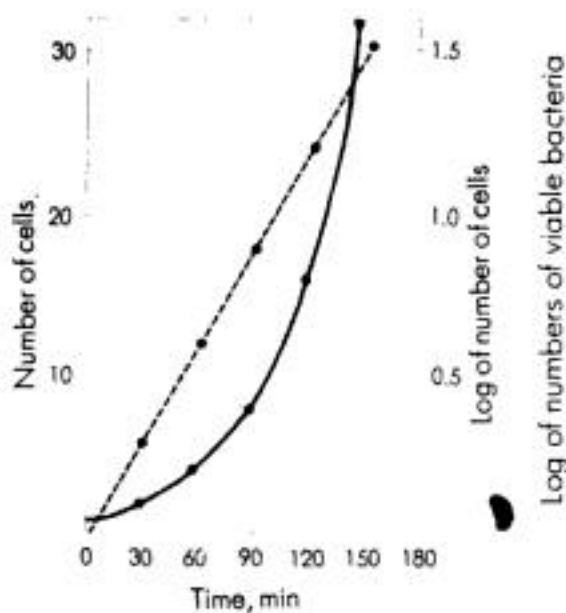
If we now substitute the value of  $\log_{10} 2$ , which is 0.301, in the above equation, we can simplify the equation to

$$\begin{aligned} n &= \frac{\log_{10} N - \log_{10} N_0}{0.301} \\ n &= 3.3 (\log_{10} N - \log_{10} N_0) \end{aligned} \quad (4)$$

Thus, by use of Eq. (4), we can calculate the number of generations that have taken place, providing we know the initial population and the population after growth has occurred.

## Normal Growth Cycle (Growth Curve) of Bacteria

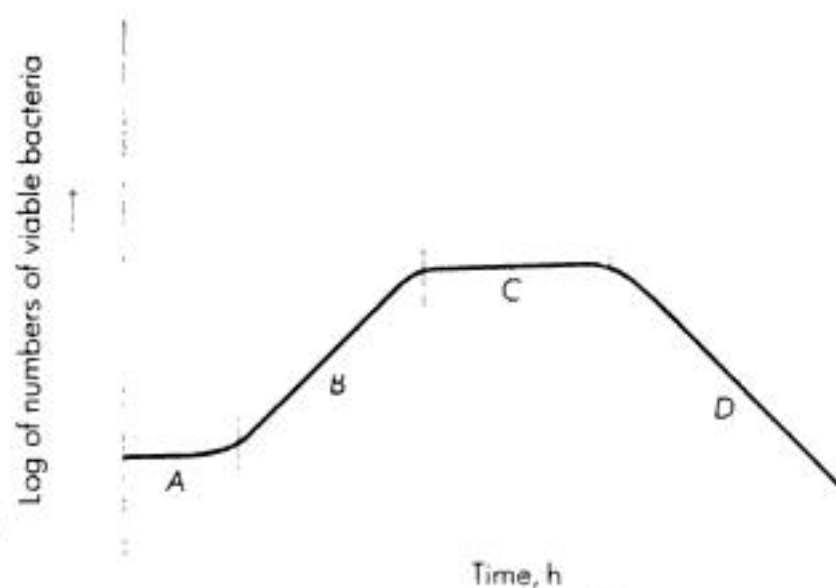
Assume that a single bacterium has been inoculated into a flask of liquid culture medium which is subsequently incubated. Eventually the bacterium will undergo binary fission and a period of rapid growth will ensue in which the cells multiply at an exponential rate. During this period of rapid growth, if we used the theoretical number of bacteria which should be present at various intervals of time and then plotted the data in two ways (logarithm of number of bacteria and arithmetic number of bacteria versus time), we would obtain the curve shown in Fig. 7-5. Here, the population increases regularly, doubling at regular



**Figure 7-5.** Hypothetical bacterial growth curve, assuming that one bacterial cell is inoculated into a medium and divisions occur regularly at 30-min intervals (generation time). — — — = logarithm of number of bacteria versus time; — = arithmetic number of bacteria versus time.

### The Lag Phase

### The Logarithmic or Exponential Phase



**Figure 7-6.** Typical bacterial growth curve. A, lag phase; B, log (logarithmic), or exponential, phase; C, stationary phase; D, death or decline phase.

time intervals (the generation time) during incubation. However, exponential growth represents only one specific portion of the growth cycle of a population. In reality, when we inoculate a fresh medium with a given number of cells, determine the bacterial population intermittently during an incubation period of 24 h (more or less), and plot the logarithms of the number of cells versus time, we obtain a curve of the type illustrated in Fig. 7-6. From this it can be seen that there is an initial period of what appears to be no growth (the lag phase), followed by rapid growth (the exponential or logarithmic phase), then a leveling off (stationary phase), and finally a decline in the viable population (death or decline phase). Between each of these phases there is a transitional period (curved portion). This represents the time required before all cells enter the new phase. Let us examine what happens to the bacterial cells during each of the phases of the growth curve.

The addition of inoculum to a new medium is not followed immediately by a doubling of the population. Instead, the population remains temporarily unchanged, as illustrated in Fig. 7-6. But this does not mean that the cells are quiescent or dormant; on the contrary, during this stage the individual cells increase in size beyond their normal dimensions. Physiologically they are very active and are synthesizing new protoplasm. The bacteria in this new environment may be deficient in enzymes or coenzymes which must first be synthesized in amounts required for optimal operation of the chemical machinery of the cell. Time for adjustments in the physical environment around each cell may be required. The organisms are metabolizing, but there is a lag in cell division.

At the end of the lag phase, each organism divides. However, since not all organisms complete the lag period simultaneously, there is a gradual increase in the population until the end of this period, when all cells are capable of dividing at regular intervals.

During this period the cells divide steadily at a constant rate, and the log of the number of cells plotted against time results in a straight line (Figs. 7-5 and 7-6). Moreover, the population is most nearly uniform in terms of chemical composition of cells, metabolic activity, and other physiological characteristics.



**Table 7-1.** Generation Times of Several Species of Bacteria

Bacterium	Medium	Temperature, °C	Generation Time, min
<i>Escherichia coli</i>	Milk	37	12.5
	Broth	37	17
<i>Bacillus thermophilus</i>	Broth	55	18.3
<i>Streptococcus lactis</i>	Milk	37	26
	Lactose broth	37	48
<i>Staphylococcus aureus</i>	Broth	37	27–30
<i>Bacillus mycoides</i>	Broth	37	28
<i>Lactobacillus acidophilus</i>	Milk	37	66–87
<i>Bradyrhizobium japonicum</i>	Mineral salts + yeast extract + mannitol	25	344–461
<i>Mycobacterium tuberculosis</i>	Synthetic	37	792–932
<i>Treponema pallidum</i>	Rabbit testes	37	1,980

SOURCE: W. B. Spector (ed.): *Handbook of Biological Data*, table 75, Saunders, Philadelphia, 1956.

The generation time  $g$  (the time required for the population to double) can be determined from the number of generations  $n$  that occur in a particular time interval  $t$ . Using Eq. (4) for  $n$ , the generation time can be calculated by the following formula:

$$g = \frac{t}{n} = \frac{t}{3.3 (\log_{10} N - \log_{10} N_0)} \quad (5)$$

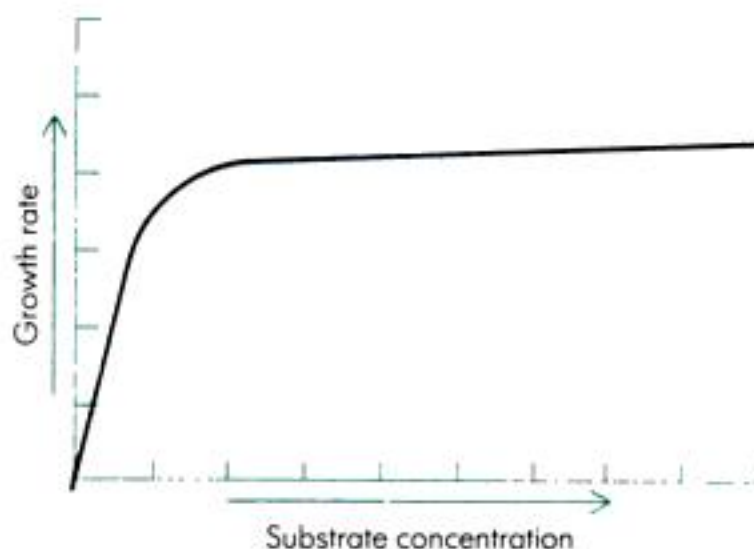
Not all bacteria have the same generation time; for some, such as *E. coli*, it may be 15 to 20 minutes; for others it may be many hours (see Table 7-1). Similarly, the generation time is not the same for a particular species under all conditions. It is strongly dependent upon the nutrients in the medium and on prevailing physical conditions, such as those outlined in Chap. 6.

During exponential growth, the growth rate (i.e., the number of generations per hour), termed  $R$ , is the reciprocal of the generation time  $g$ . It is also the slope of the straight line obtained when the log number of cells is plotted against time:

$$R = \frac{3.3(\log_{10} N - \log_{10} N_0)}{t} \quad (6)$$

You may ask how this growth rate can remain constant during the logarithmic phase of growth even though the concentration of substrate (i.e., some essential nutrient in the culture medium, usually the carbon and energy source) is continually decreasing through utilization by the organisms. To understand this, one must recognize that the relationship between  $R$  and substrate concentration is not a simple linear relationship, as shown in Fig. 7-7. When the substrate concentration is high, a change in the concentration has very little effect on the growth rate. It is only when the substrate concentration becomes quite low that the growth rate begins to decrease significantly. Since bacteria are commonly "overfed" in laboratory culture, (i.e., are supplied with far greater substrate concentrations than they need), they can multiply at a constant exponential rate for many generations before the substrate level becomes low enough to affect this rate.

**Figure 7-7.** The effect of nutrient (substrate) concentration upon the growth rate of a bacterial culture. The level of substrate commonly provided in a bacterial culture is sufficiently high (right portion of curve) so that, even though the bacteria use up some substrate during the log phase of growth, the growth rate does not decrease appreciably. It is only when substrate levels become very low (left portion of curve) that the growth rate begins to be severely affected.



A microbiologist must be able to calculate growth rates and generation times. For example, it is often essential to predict how long it will take a certain population to grow to a given level. An appreciation of the full meaning of the normal growth curve is also necessary; it must be understood that during some phases of growth the cells are young and actively metabolizing while during others they are dying, so that there may be enormous structural and physiological differences between cells harvested at different times. Physical conditions and chemical substances may also affect organisms differently during different phases. Because, in general, cells in the logarithmic phase of growth are the most uniform and are in a more clearly defined condition than in any other phase, log-phase cultures are commonly used for studies of microbial metabolism.

### The Stationary Phase

The logarithmic phase of growth begins to taper off after several hours, again in a gradual fashion represented by the transition from a straight line through a curve to another straight line, the stationary phase, as shown in Fig. 7-6. This trend toward cessation of growth can be attributed to a variety of circumstances, particularly the exhaustion of some nutrients, and, less often, the production of toxic products during growth. The population remains constant for a time, perhaps as a result of complete cessation of division or perhaps because the reproduction rate is balanced by an equivalent death rate.

### The Phase of Decline or Death

Following the stationary phase the bacteria may die faster than new cells are produced, if indeed some cells are still reproducing. Undoubtedly a variety of conditions contribute to bacterial death, but the most important are the depletion of essential nutrients and the accumulation of inhibitory products, such as acids. During the death phase, the number of viable cells decreases exponentially, essentially the inverse of growth during the log phase. Bacteria die at different rates, just as they grow at different rates. Some species of Gram-negative cocci die very rapidly, so that there may be very few viable cells left in a culture after 72 h or less. Other species die so slowly that viable cells may persist for months or even years.

### Transitional Periods Between Growth Phases

Note that a culture proceeds gradually from one phase of growth to the next (Fig. 7-6). This means that not all the cells are in an identical physiological



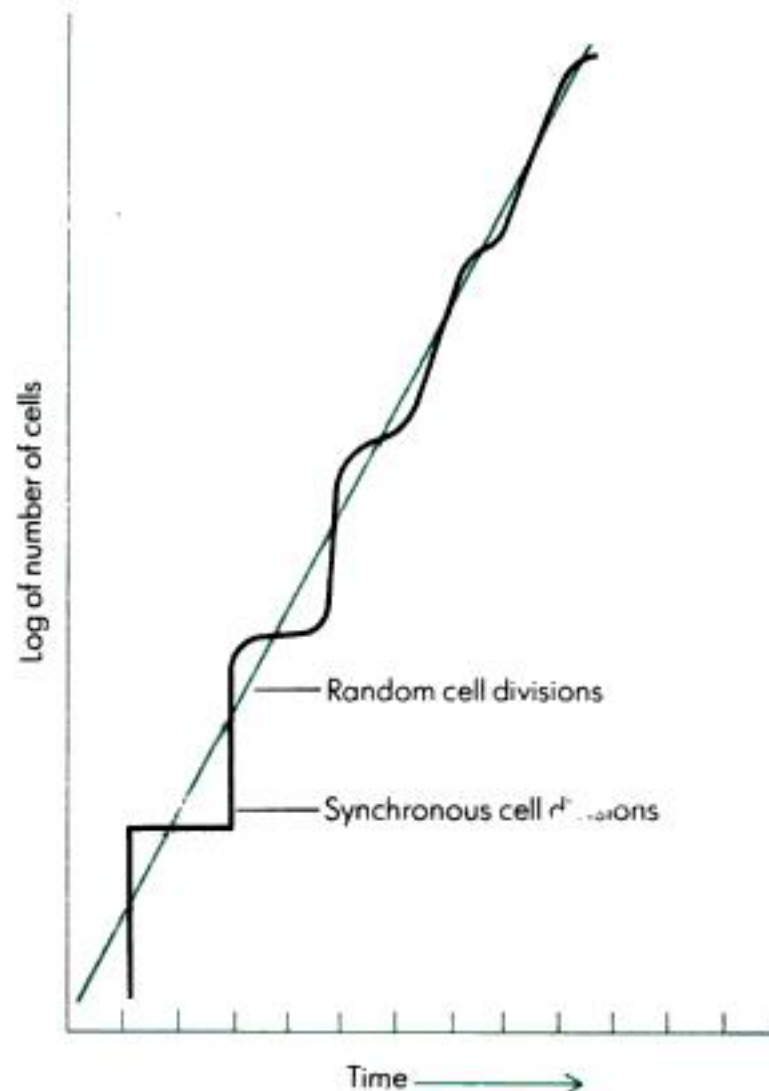
condition toward the end of a given phase of growth. Time is required for some to catch up with others.

## Synchronous Growth

There are many aspects of microbiological research where it would be desirable to relate the various aspects of bacterial growth, organization, and differentiation to a particular stage of the cell division cycle. It is not feasible to analyze a single bacterium because of its small size. However, if all the cells in a culture were to be in the same stage of the division cycle, the result from analysis of the cell crop could be interpreted as that for a single cell. There are several laboratory techniques by which we can manipulate the growth of cultures so that all the cells will be in the same stage of their growth cycle, i.e., growing **synchronously**.

The synchrony generally lasts only a few generations, since even the daughters of a single cell soon get out of phase with one another. A population can be synchronized by manipulating the physical environment or the chemical composition of the medium. For example, the cells may be inoculated into a medium at a suboptimal temperature; if they are kept in this condition for some time, they will metabolize slowly but will not divide. When the temperature is subsequently raised, the cells will undergo a synchronized division. The most common method of synchronization takes advantage of the fact that the smallest cells in a log-phase culture are those which have just divided. When these cells are separated out by filtration or by differential centrifugation, they are reasonably well synchronized with each other. Figure 7-8 shows the growth pattern of a population of synchronous cells.

**Figure 7-8.** Synchronous growth of bacteria. The steplike growth pattern indicates that all the cells of the population divide at about the same time.



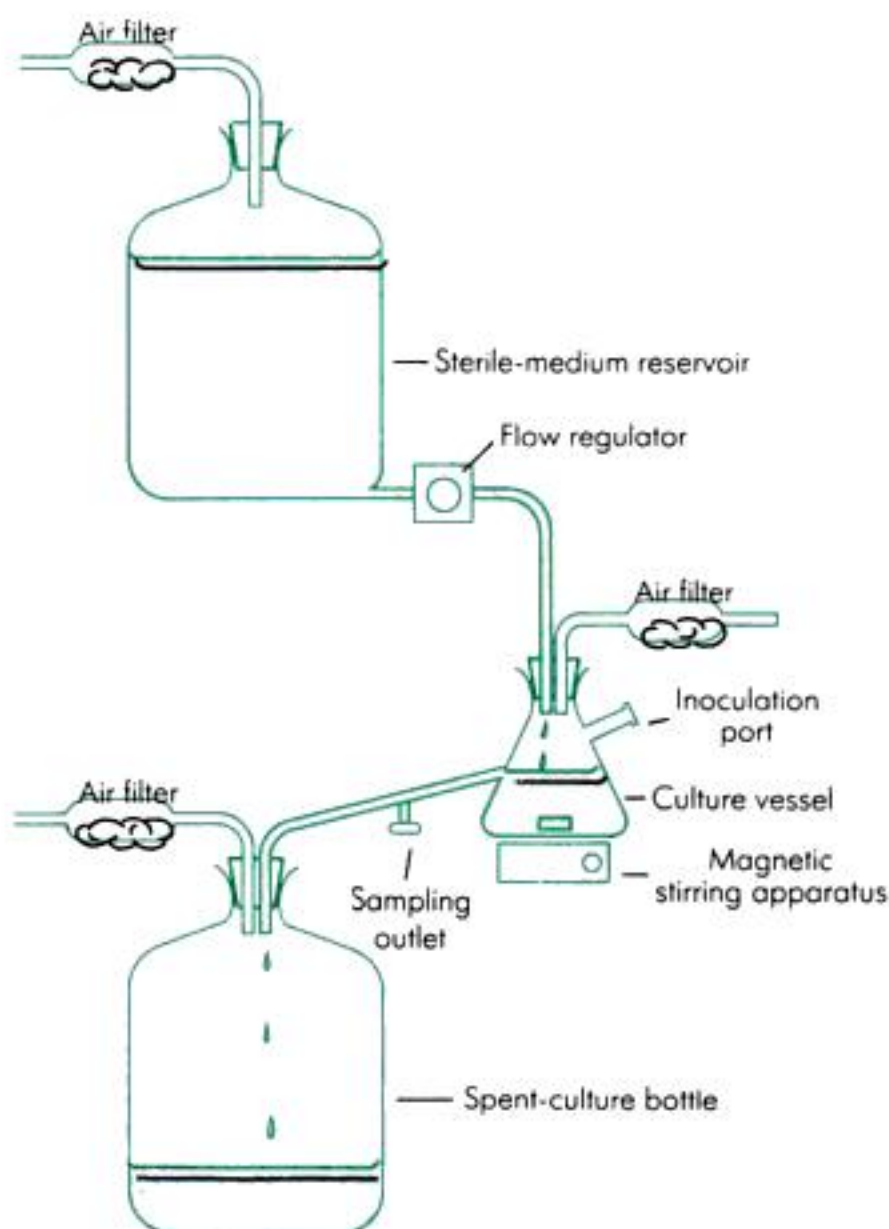


## Continuous Culture

In both experimental research and in industrial processes, it is often desirable to maintain a bacterial population growing at a particular rate in the exponential or log phase. This condition is known as **steady-state** growth. The culture volume and the cell concentration are both kept constant by allowing fresh sterile medium to enter the culture vessel at the same rate that "spent" medium, containing cells, is removed from the growing culture (see Fig. 7-9). Under these conditions, the rate at which new cells are produced in the culture vessel is exactly balanced by the rate at which cells are being lost through the overflow from the culture vessel.

One type of system that is widely employed for continuous cultivation is the **chemostat**. This system depends on the fact that the concentration of an essential nutrient (substrate) within the culture vessel will control the growth rate of the cells. The concentration of substrate within the culture vessel is in turn controlled by the dilution rate, i.e., the rate at which fresh medium is being added to the culture (flow rate) divided by the volume of the culture vessel. Therefore, by adjusting the dilution rate we can control the growth rate. For example, suppose that the dilution rate is very low. The cells reach a high density because they are leaving the culture vessel at a very slow rate; moreover, they have time to use the substrate almost completely. Therefore, the substrate concentration is maintained at a low level within the vessel. This low substrate concentration permits the cells to grow at only a slow rate. On the other hand, if the dilution rate is high, the cell density is low because the cells are leaving the vessel at a

**Figure 7-9.** Apparatus for continuous cultivation of bacteria. The system can be regulated for continuous addition of fresh sterile medium to and removal of spent medium (and cells) from the culture vessel.





high rate; moreover, they have little time to utilize the substrate that is entering the vessel, and therefore the substrate concentration is maintained at a high level within the vessel (but still less than that in the sterile-medium reservoir). This high concentration allows the cells to grow at a high rate. In each case the growth rate automatically adjusts to match the dilution rate. However, if the dilution rate is increased to the point where it exceeds the maximum growth rate of the cells, then **washout** occurs; that is, the cells cannot grow as fast as the rate at which the culture is being diluted by fresh medium, and they are soon eliminated from the culture vessel.

A second type of continuous culture apparatus is the **turbidostat**. Here a photoelectric device continuously monitors the cell density within the culture vessel and controls the dilution rate to maintain the cell density at a constant value. If the density becomes too high the dilution rate is increased; if the density becomes too low, the dilution rate is decreased.

## **QUANTITATIVE MEASUREMENT OF BACTERIAL GROWTH**

We have seen that the term growth as commonly applied in microbiology refers to the magnitude of the total population. Growth in this sense can be determined by numerous techniques based on one or more of the following types of measurement:

- 1 **Cell count.** Directly by microscopy or by using an electronic particle counter, or indirectly by a colony count
- 2 **Cell mass.** Directly by weighing or by a measurement of cell nitrogen, or indirectly by turbidity
- 3 **Cell activity.** Indirectly by relating the degree of biochemical activity to the size of the population

Certain specific procedures will illustrate the application of each type of measurement.

### **Direct Microscopic Count**

Bacteria can be counted easily and accurately with the Petroff-Hausser counting chamber. This is a special slide accurately ruled into squares that are  $1/400$  mm<sup>2</sup> in area; a glass cover slip rests  $1/50$  mm above the slide, so that the volume over a square is  $1/20,000$  mm<sup>3</sup> ( $1/20,000,000$  cm<sup>3</sup>). A suspension of unstained bacteria can be counted in the chamber, using a phase-contrast microscope. If, for example, an average of five bacteria is present in each ruled square, there are  $5 \times 20,000,000$ , or  $10^8$ , bacteria per milliliter. Direct microscopic counts can be made rapidly and simply with a minimum of equipment; moreover, the morphology of the bacteria can be observed as they are counted. Very dense suspensions can be counted if they are diluted appropriately; however, suspensions having low numbers of bacteria, e.g., at the beginning of a growth curve, cannot be counted accurately.

### **Electronic Enumeration of Cell Numbers**

In this method, the bacterial suspension is placed inside an electronic particle counter, within which the bacteria are passed through a tiny orifice 10 to 30  $\mu$ m in diameter. This orifice connects the two compartments of the counter which contain an electrically conductive solution. As each bacterium passes through the orifice, the electrical resistance between the two compartments increases momentarily. This generates an electrical signal which is automatically



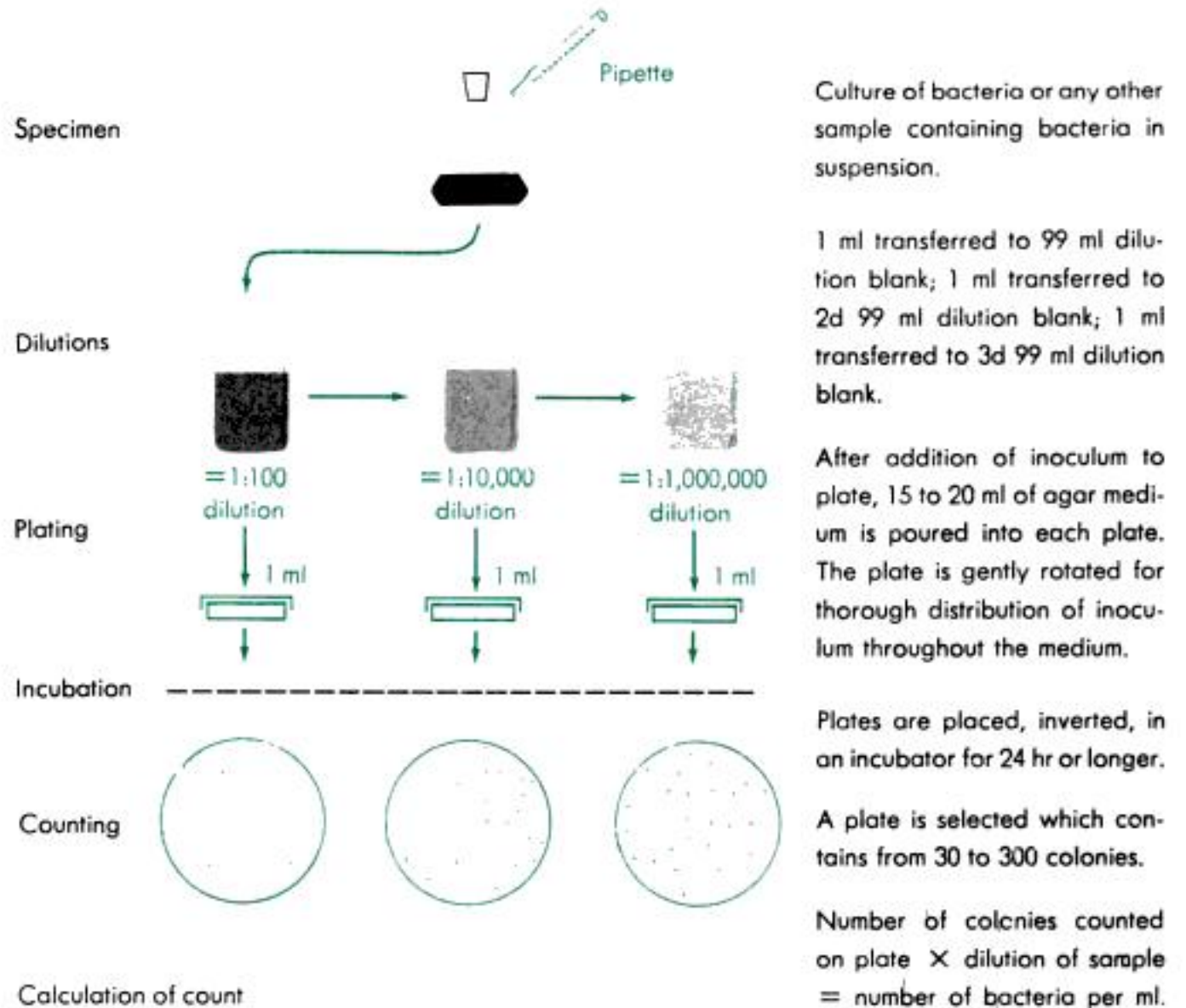
counted. Although this method is rapid, it requires sophisticated electronic equipment; moreover, the orifice tends to become clogged.

The main disadvantage of direct counting of cell numbers is that there is no way to determine whether the cells being counted are viable. To determine the viable count of a culture, we must use a technique that allows viable cells to multiply, such as the plate-count method or the membrane-filter method.

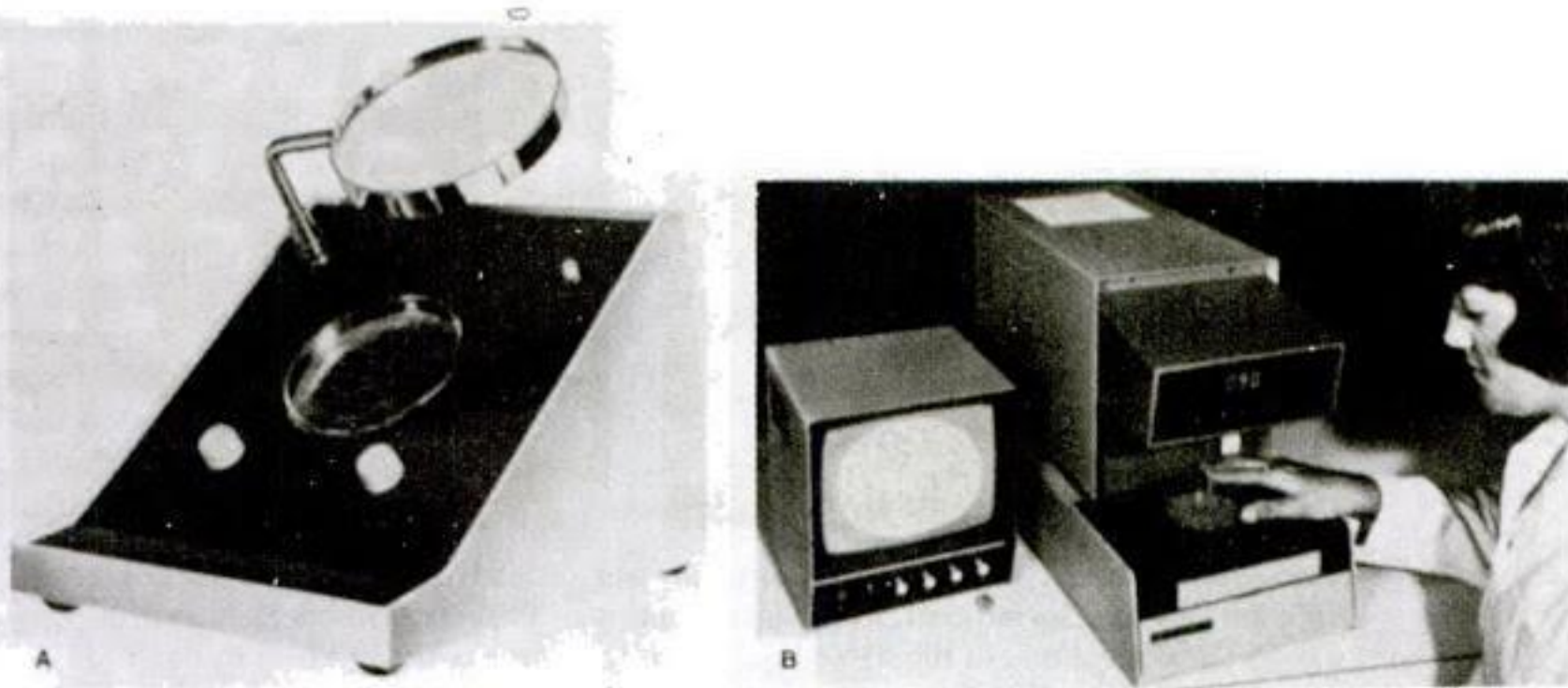
### The Plate-Count Method

This method, illustrated in Fig. 7-10, allows determination of the number of cells that will multiply under certain defined conditions. A measured amount of the bacterial suspension is introduced into a Petri dish, after which the agar medium (maintained in liquid form at 45°C) is added and the two thoroughly mixed by rotating the plate. When the medium solidifies, the organisms are trapped in the gel. Each organism grows, reproducing itself until a visible mass of organisms—a colony—develops; i.e., one organism gives rise to one colony. Hence, a colony count performed on the plate reveals the viable microbial population of the inoculum. The original sample is usually diluted so that the number of colonies developing on the plate will fall in the range of 30 to 300. Within this range the count can be accurate, and the possibility of interference of the growth of one organism with that of another is minimized. Colonies are usually counted by illuminating them from below (dark-field illumination) so that they are easily visible, and a large magnifying lens is often used (see Fig. 7-11A). Various electronic techniques have been developed for the counting of colonies (Fig. 7-11B).

**Figure 7-10.** The plate-count technique, in which the sample is diluted quantitatively and measured amounts of the dilutions are cultured in Petri dishes.







**Figure 7-11.** Bacterial colony counters. (A) Quebec colony counter. A Petri dish fits into the recess in the platform. The Petri dish is illuminated from beneath while the lens provides X1.5 magnification. (Courtesy of American Optical Corporation.) (B) An electronic colony counter. The Petri dish is placed on the illuminated stage, the count bar is depressed, and the precise number of colonies is instantly displayed on a digital readout. (Courtesy of New Brunswick Scientific Company, Inc.)

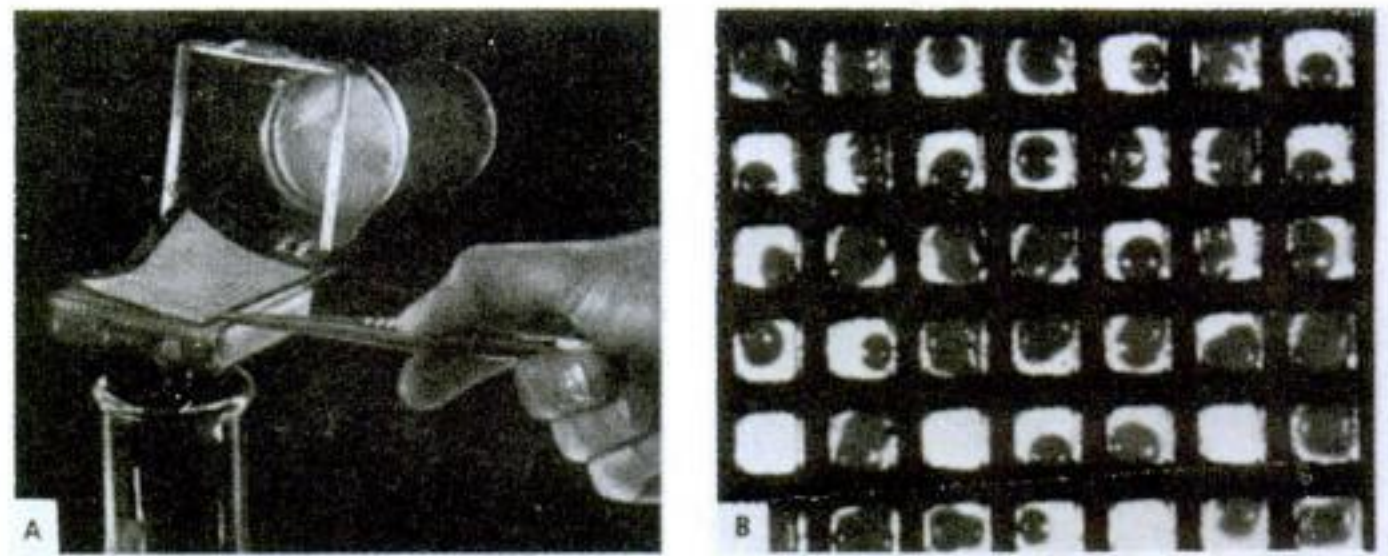
One limitation of the plate-count technique is that the only bacteria that will be counted are those which can grow on the medium used and under the conditions of incubation provided. This can be an important consideration if a mixture of bacteria is to be counted. Another limitation is that each viable organism that is capable of growing under the culture conditions provided may not necessarily give rise to one colony. The development of one colony from one cell can occur when the bacterial suspension is homogeneous and no aggregates of cells are present; however, if the cells have a tendency to aggregate, e.g., cocci in clusters (staphylococci), chains (streptococci), or pairs (diplococci), the resulting counts will be lower than the number of individual cells. For this reason the "counts" are often reported as **colony-forming units** per milliliter rather than number of bacteria per milliliter.

The plate-count technique is used routinely and with satisfactory results for the estimation of bacterial populations in milk, water, foods, and many other materials. It is easy to perform and can be adapted to the measurement of populations of any magnitude. It has the advantage of sensitivity, since very small numbers of organisms can be counted. Theoretically, if a specimen contains as few as one bacterium per milliliter, one colony should develop upon the plating of 1 ml.

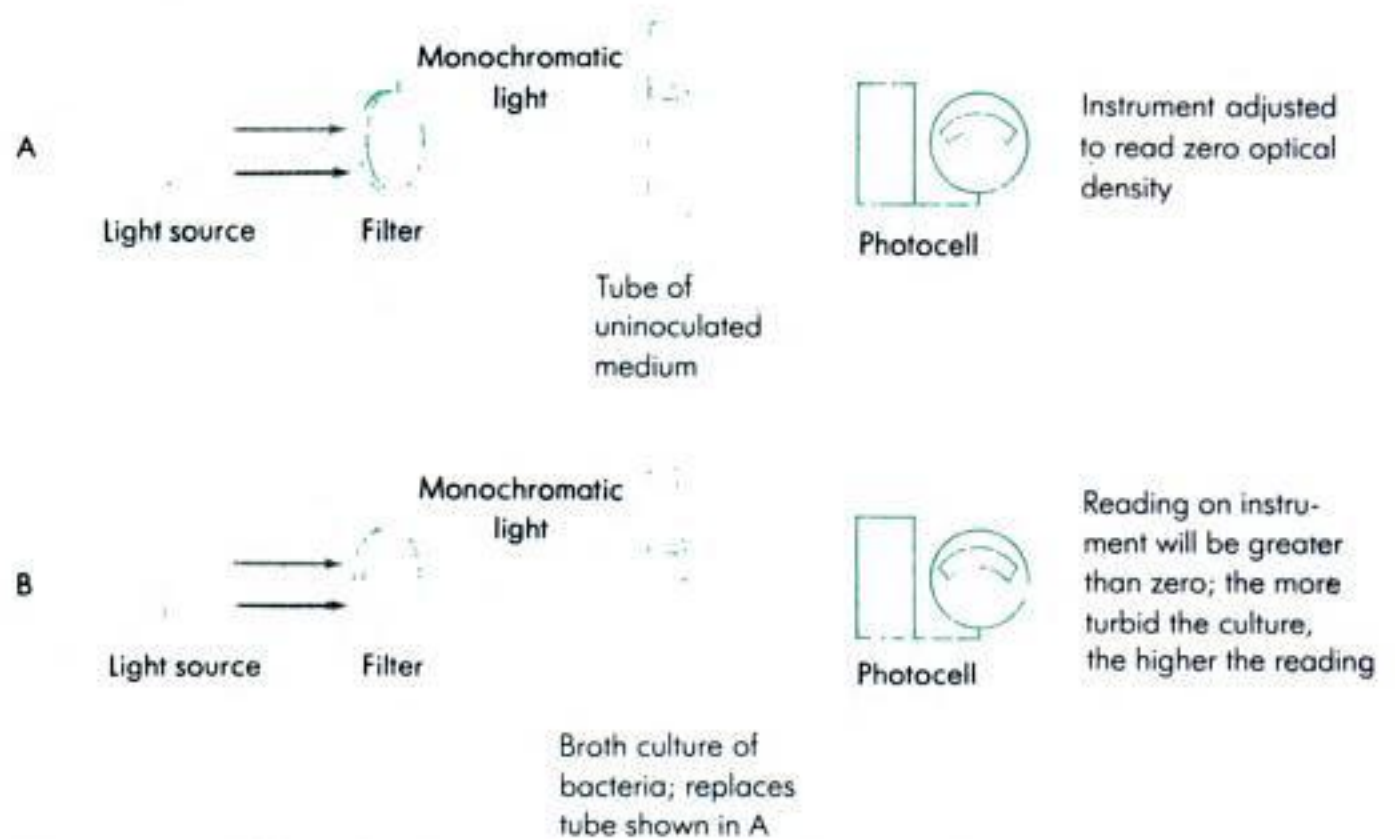
### Membrane-Filter Count

A very useful variation on the plate-count technique is based on the use of molecular or membrane filters. These filters have a known uniform porosity of predetermined size sufficiently small to trap microorganisms. This technique is particularly valuable in determining the number of bacteria in a large sample that has a very small number of viable cells; e.g., the bacteria in a large volume of air or water can be collected simply by filtering them through an assembly as illustrated in Fig. 7-12A. The membrane, with its trapped bacteria, is then placed in a special plate containing a pad saturated with the appropriate medium. Special media and dyes can be used to make it easier to detect certain types of organisms than with the conventional plate count. During incubation,





**Figure 7-12.** (A) Filtration apparatus for use with a membrane filter. After placing the filter on the support, the hinged upper part of the apparatus clamps it in place. A known volume of the bacteria-containing sample is then passed through the filter. (B) The filter is then incubated on a suitable culture medium. In the particular type of filter shown, a grid divides the filter into 1,600 small square compartments, and colony growth is restricted to these compartments. This greatly facilitates the counting of the colonies. (Courtesy of New Brunswick Scientific Company, Inc.)



**Figure 7-13.** Schematic illustration of the use of a photoelectric colorimeter for measuring bacterial populations. The instrument measures optical density (also termed absorbance), a function of light intensity which is almost linearly proportional to cell mass. (A) Adjustment of instrument. A glass tube (cuvette) filled with uninoculated culture medium is used to set the instrument to give a basal optical density reading of 0. (B) The "blank" cuvette is replaced by a similar cuvette containing the broth culture (i.e., medium + cells), and the increase in optical density is recorded.



the organisms grow into colonies which appear on the membrane surface (see Fig. 7-12B).

### **Turbidimetric Methods**

Anyone who has tried to see through a fog realizes that visibility is reduced in proportion to the density of the fog and the distance between the observer and the object that he or she is looking at. This is because each droplet of water in the fog absorbs and scatters the light passing through it, and the more droplets in the light path, the less one can see. Similarly, bacteria in a suspension absorb and scatter the light passing through them, so that a culture of more than  $10^7$  to  $10^8$  cells per milliliter appears **turbid** to the naked eye. A spectrophotometer or colorimeter can be used for **turbidimetric measurements** of cell mass (see Fig. 7-13). Turbidimetry is a simple, rapid method for following growth; however, the culture to be measured must be dense enough to register some turbidity on the instrument. Moreover, it may not be possible to measure cultures grown in deeply colored media or cultures that contain suspended material other than bacteria. It must also be recognized that dead as well as living cells contribute to turbidity.

### **Determination of Nitrogen Content**

The major constituent of cell material is protein, and since nitrogen is a characteristic part of proteins, one can measure a bacterial population or cell crop in terms of bacterial nitrogen. Bacteria average approximately 14 percent nitrogen on a dry-weight basis, although this figure is subject to some variation introduced by changes in cultural conditions or differences between species. To measure growth by this technique, you must first harvest the cells and wash them free of medium and then perform a quantitative chemical analysis for nitrogen. Bacterial nitrogen determinations are somewhat laborious and can be performed only on specimens free of all other sources of nitrogen. Furthermore, the method is applicable only for concentrated populations. For these and other reasons, this procedure is used primarily in research.

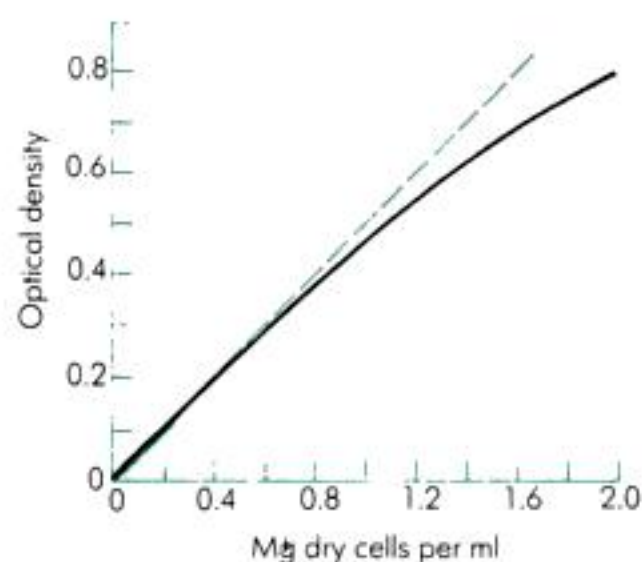
### **Determination of the Dry Weight of Cells**

This is the most direct approach for quantitative measurement of a mass of cells. However, it can be used only with very dense suspensions, and the cells must be washed free of all extraneous matter. Moreover, dry weight may not always be indicative of the amount of living material in cells. For example, the intracellular reserve material poly- $\beta$ -hydroxybutyrate can accumulate in *Azotobacter beijerinckii* at the end or the log phase of growth and during the stationary phase and finally can comprise up to 74 percent of the dry weight of the cells; thus, the dry weight may continue to increase without corresponding cell growth. Yet, for many organisms the determination of dry weight is an accurate and reliable way to measure growth and is widely used in research.

### **Measurement of a Specific Chemical Change Produced on a Constituent of the Medium**

As an example of this method of estimating cell mass, we may take a species that produces an organic acid from glucose fermentation. The assumption is that the amount of acid produced, under specified conditions and during a fixed period of time, is proportional to the magnitude of the bacterial population. Admittedly, the measurement of acid or any other end product is a very indirect approach to the measurement of growth and is applicable only in special circumstances.





**Figure 7-14.** Turbidity of a culture serves as a convenient indirect measure of the dry weight of bacterial cells in the culture, as shown in this typical curve ( — ); however, some deviation from a theoretical linear relationship ( - - - ) does occur, particularly at optical densities greater than 0.4.

### The Relation of Turbidity Measurements to Direct Expressions of Growth

It is frequently desirable to relate measurements of growth made by an indirect method, e.g., turbidity, to a direct measurement, e.g., dry weight of the cell crop. This can be done conveniently by measuring the bacterial suspension simultaneously by the two methods and establishing a relationship between the values obtained, as in the following example. Samples are removed from a cell suspension and dried under predetermined conditions, and the weight of cells per milliliter is determined. From the same cell suspension, dilutions are prepared and turbidity measurements are made. We can calculate the weight of the bacteria in each dilution, since the weight of cells per milliliter of the original sample was determined. Two sets of data will be obtained, which can then be plotted (cell weight against turbidity), as illustrated in Fig. 7-14, to obtain a **standard curve**. For practical purposes, and within a certain range of concentrations, a nearly linear relationship exists. When the standard curve has been

**Table 7-2.** Summary of Methods for Measuring Bacterial Growth

Method	Some Applications	Manner in Which Growth Is Expressed
Microscopic count	Enumeration of bacteria in vaccines and cultures	Number of cells per ml
Electronic enumeration	Same as for microscopic count	Same as for microscopic count
Plate count	Enumeration of bacteria in milk, water, foods, soil, cultures, etc.	Colony-forming units per ml
Membrane filter	Same as plate count	Same as plate count
Turbidimetric measurement	Microbiological assay, estimation of cell crop in broth, cultures, or aqueous suspensions	Optical density (absorbance)
Nitrogen determination	Measurement of cell crop from heavy culture suspensions to be used for research in metabolism	Mg nitrogen per ml
Dry weight determination	Same as for nitrogen determination	Mg dry weight of cells per ml
Measurements of biochemical activity, e.g., acid production by cultures	Microbiological assays	Milliequivalents of acid per ml or per culture



established, we can measure the turbidity of a bacterial suspension and convert this value to bacterial weight. Similarly, we can prepare a standard curve correlating other direct measurements (numbers of bacteria or bacterial nitrogen) with turbidity. Thus it is possible to use the convenient indirect measurement (turbidity) and convert the value to a direct expression of growth.

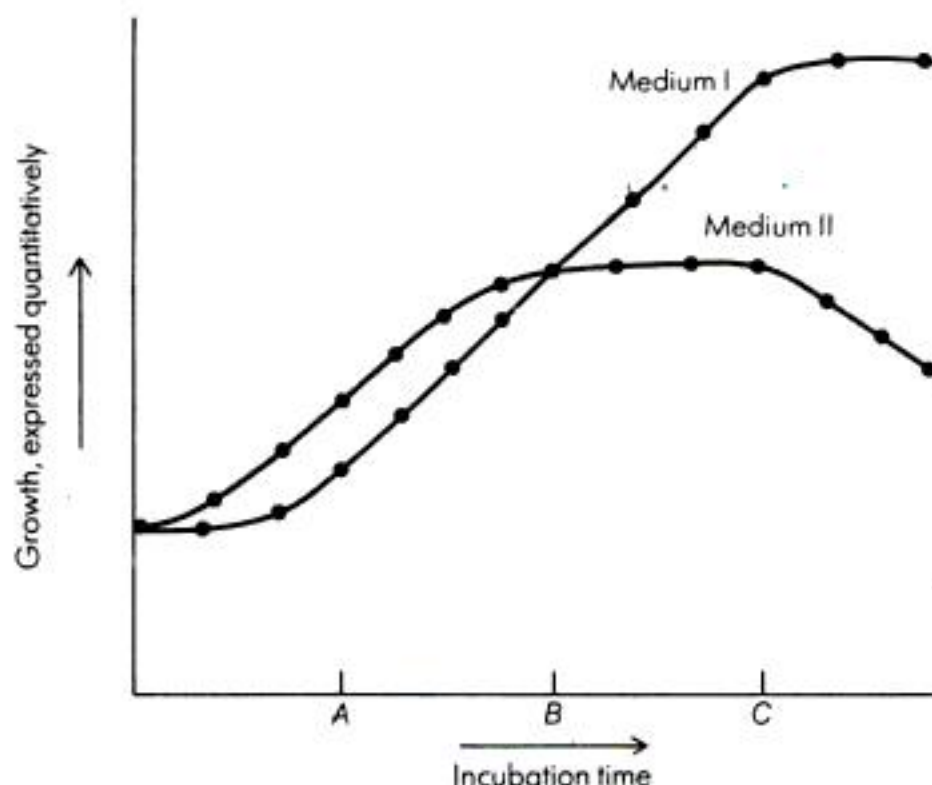
### The Selection of a Procedure to Measure Growth

Table 7-2 summarizes the methods described above for measuring bacterial growth. Each has its particular advantages and limitations, and no one method can be recommended universally. The best procedure for your work can be selected only after these factors are considered in relation to the problem at hand. The colony count is the most widely used procedure for general microbiological work, and complete familiarity with this technique, both in principle and practice, is essential. It should be emphasized that the colony count is theoretically the only technique that reflects the viable population. Furthermore, it is not at all unlikely that discrepancies may occur in results of growth of a bacterial population when measured by two different methods. For example, a microscopic count of a culture in the stationary phase would include all cells, viable and nonviable, whereas the colony count would reveal only the viable population.

### Importance of Quantitative Measurement of Growth

Before we can evaluate or interpret growth responses of bacteria in different media or under various conditions, growth must be expressed in quantitative terms. In microbiology the term growth is used in several ways. For example, we may judge a certain set of conditions as being good because the bacteria grow rapidly, but the final total cell crop may not be as large as under another set of conditions where the growth proceeds at a slower rate but continues to increase over a longer time period. Such a situation is shown schematically in Fig. 7-15, where the growth of the same bacterial species is compared in two different media. If we measured growth at time A, we should conclude that growth is best in medium II; measured at time B, growth would be equally good in both media; and at time C, growth would be better in medium I. If we were primarily interested in a large cell crop, we should select medium I. In any

**Figure 7-15.** Quantitative measurement of growth is significant for interpretation of various growth responses. Hypothetical growth response of same bacterium in media of two different compositions. Compare the cell crops, or amount of growth, at times A, B, and C.





event, we must have knowledge of growth in quantitative terms to make the correct choice.

## QUESTIONS

- 1 How does the term growth as used in microbiology differ from the same term as applied to higher plants and animals?
- 2 Describe the differences between the various modes of cell division in bacteria.
- 3 How is it that the septum formation in *Streptococcus faecalis* accounts for all the new cell wall material of newly formed daughter cells, yet in *Bacillus subtilis* it accounts for only 15 percent?
- 4 In a culture of bacteria that is actively multiplying by transverse binary fission, are there any "old" cells? Explain.
- 5 During log-phase growth of a bacterial culture, a sample is taken at 8:00 a.m. and found to contain 1,000 cells per milliliter. A second sample is taken at 5:54 p.m. and is found to contain 1,000,000 cells per milliliter. What is the generation time in hours?
- 6 Would you expect generation time to be a constant characteristic of a bacterial species? Explain.
- 7 In the lag phase of growth the number of bacteria remains constant. Does this mean the cells are dormant and inert? Explain.
- 8 Draw a typical bacterial growth curve and label the various phases. Discuss those factors which determine the beginning and end of each phase.
- 9 When the appropriate data are plotted for the log-phase growth of a bacterial culture, how is it that we obtain a steady increase in the number of cells rather than a series of stepwise increases? Explain.
- 10 How can synchronous growth of a bacterial culture be obtained? In what way could a synchronously growing culture be useful for the electron microscopist who is trying to determine the cytological changes associated with bacterial growth?
- 11 What is steady-state growth and what advantages does it offer? Describe how steady-state growth at a certain rate could be obtained by use of a chemostat.
- 12 Compare the direct and indirect methods for estimating bacterial populations on the basis of (a) practical applications, (b) advantages and (c) limitations of use.

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## Chapter 8

# Pure Cultures and Cultural Characteristics

### OUTLINE Natural Microbial Populations (Mixed Cultures)

#### Selective Methods

Chemical Methods of Selection • Physical Methods of Selection • Biological Methods of Selection • Selection in Nature

#### Pure Cultures

Methods of Isolating Pure Cultures

#### Maintenance and Preservation of Pure Cultures

Methods of Maintenance and Preservation • Culture Collections

#### Cultural Characteristics

Colony Characteristics • Characteristics of Broth Cultures

In natural environments a particular type of bacterium, i.e., a bacterial species, usually occurs as only one member of a large and complex population containing many other species. To study the characteristics of one species, that species must be separated from all the other species, i.e., it must be **isolated** in pure culture. However, before attempting isolation, it is often helpful to use a **selective method** first. Such a method can increase the relative proportion of the desired species in the population so that it can be more easily isolated. Once obtained, a pure culture can be maintained or preserved in a **culture collection**. Different species of bacteria growing on the same kind of medium may appear quite different; thus knowledge of the appearance, or the **cultural characteristics**, of a species is useful for the recognition of certain kinds of bacteria and may also serve as an aid to the identification of species. In this chapter we shall describe methods for selection, isolation, and preservation of bacteria, as well as their cultural characteristics on various media.

### NATURAL MICROBIAL POPULATIONS (MIXED CULTURES)

The microbial population in our environment is large and complex. Many different microbial species normally inhabit various parts of our bodies, such as the oral cavity, the intestinal tract, and the skin. These microbes may be present in extremely large numbers. For example, a single sneeze may disperse from 10,000 to 100,000 bacteria. One gram of feces may contain  $10^{11}$  bacteria. Our environment—air, soil, water—likewise consists of mixed populations of



bacteria plus other microbes. In fertile garden soil, microorganisms may number several billions per gram and include many species of bacteria, fungi, algae, and protozoa. A study of the microorganisms in these habitats requires knowledge of the specific microbes present. This, in turn, requires unraveling the complex mixed population into pure cultures of separate, distinct species.

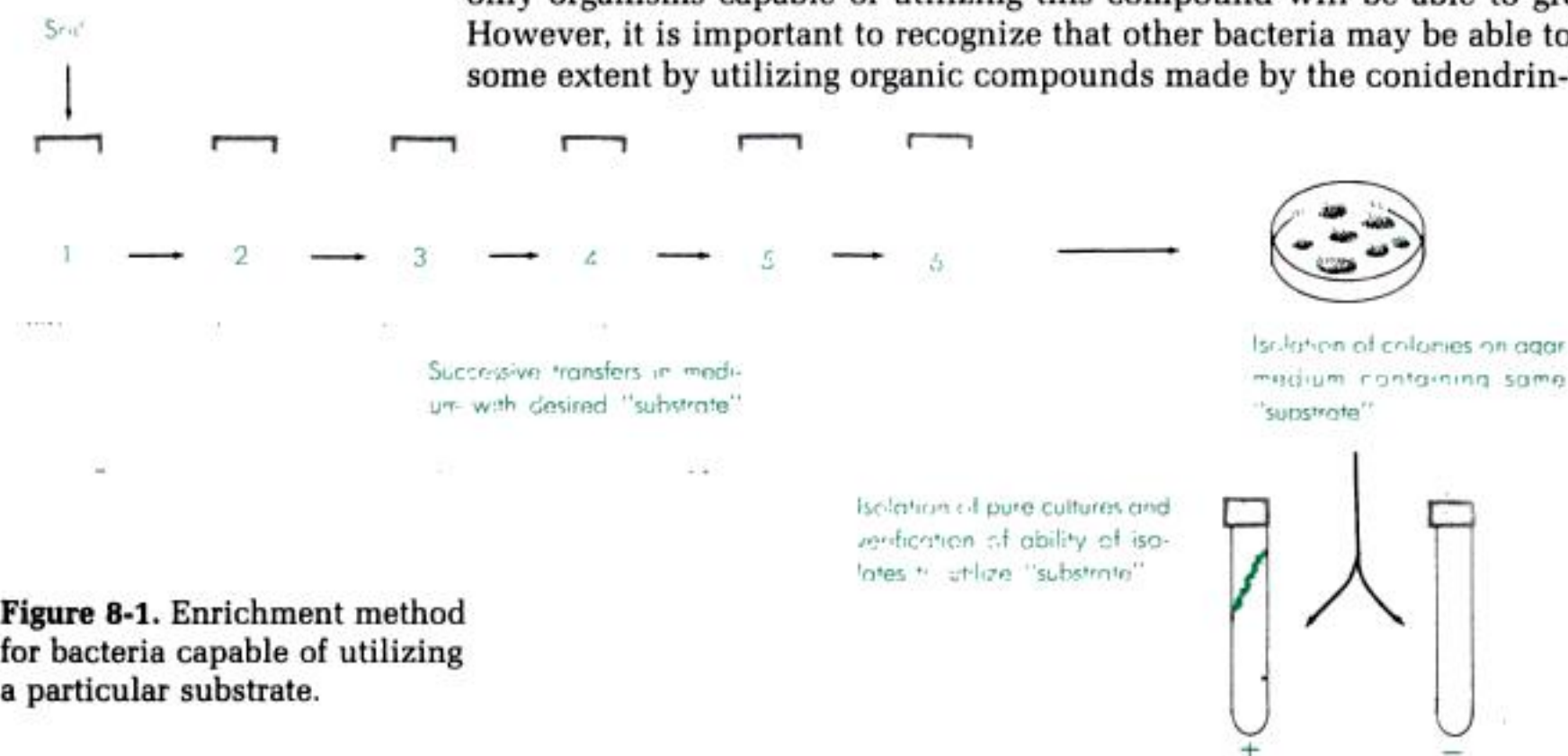
## SELECTIVE METHODS

A particular bacterial species is often present in small numbers compared to the total population of a mixed culture. Moreover, the species may be one that grows less rapidly on ordinary culture media than other species. In order to achieve its isolation into pure culture, it is helpful—and often necessary—to first achieve an increase in the relative number of the species, preferably to the point where the species becomes the numerically dominant component of the population. This can be accomplished by the use of selective methods. These methods favor the growth of the desired species while discouraging, or even killing, the other organisms present in the mixed culture. Chemical, physical, or biological methods are used in order to achieve selection of a particular kind of bacterium.

### Chemical Methods of Selection

#### Use of a Special Carbon or Nitrogen Source

One type of chemical method is to provide in the culture medium a substrate, i.e., a single carbon or nitrogen source, that can be used only by the species being sought (Fig. 8-1). This particular kind of selection is often referred to by a special name, **enrichment**. For example, if we wish to isolate, from soil, bacteria capable of utilizing a very complex organic compound like  $\alpha$ -conidrin, a constituent of wood, we find that when we inoculate a medium such as nutrient agar directly with the soil sample, our chances of finding  $\alpha$ -conidrin-utilizing bacteria will be very limited. There are so many other rapidly growing bacteria present that the more slowly growing kind we wish to obtain will be soon overgrown. Consequently, we prepare a liquid **enrichment medium** in which  $\alpha$ -conidrin is the sole source of carbon. Under these conditions, only organisms capable of utilizing this compound will be able to grow well. However, it is important to recognize that other bacteria may be able to grow to some extent by utilizing organic compounds made by the conidrin-utilizing



**Figure 8-1.** Enrichment method for bacteria capable of utilizing a particular substrate.



organisms and that the method is not completely specific. As another example, if we wish to select for nitrogen-fixing bacteria, nitrogen gas ( $N_2$ ) can be supplied as the sole nitrogen source, since only nitrogen-fixing bacteria will be able to grow well. Other bacteria may grow, but to a lesser degree, by using the nitrogenous products made by the nitrogen-fixers.

### Use of Dilute Media

Certain aquatic bacteria, such as *Caulobacter* species, are capable of growing with very low levels of carbon or nitrogen sources. Consequently, one way to select for such bacteria is to inoculate a mixed culture into a very dilute medium, e.g., a broth containing only 0.01 percent peptone. The medium must have low enough levels of nutrients that other kinds of organisms will not be able to grow well in it.

### Use of Inhibitory or Toxic Chemicals

The addition of low levels of certain chemicals, such as dyes, bile salts, salts of heavy metals, or antibiotics, to culture media can be useful for the selection of certain kinds of bacteria. The following are examples of this type of selection:

- 1 Many Gram-negative bacteria can grow in the presence of low concentrations of various dyes that inhibit the growth of Gram-positive bacteria. Similarly, intestinal bacteria can grow in the presence of bile salts such as sodium deoxycholate, whereas nonintestinal bacteria are usually inhibited. Consequently, a medium containing crystal violet dye plus sodium deoxycholate will allow Gram-negative intestinal bacteria to grow but will inhibit most other kinds of bacteria. An example of such a medium is MacConkey agar, which is widely used to select for Gram-negative intestinal pathogens such as *Salmonella* and *Shigella* species.
- 2 *Campylobacter jejuni* is a frequent cause of diarrhea in humans, yet diarrheic stool samples contain many other kinds of bacteria that interfere with the isolation of this species. By incorporating certain antibiotics or other chemotherapeutic agents, such as vancomycin, polymyxin, and trimethoprim, into the culture medium, most of these contaminants can be inhibited without affecting the growth of *C. jejuni*.

### Physical Methods of Selection

#### Heat Treatment

To select for endospore-forming bacteria, a mixed culture can be heated to 80°C for 10 min before being used to inoculate culture media. Vegetative cells will be killed by this treatment but endospores will survive and subsequently germinate and grow.

#### Incubation Temperature

To select for psychrophilic or psychrotrophic bacteria, cultures are incubated at low temperatures, e.g., 0 to 5°C. For selection of thermophiles, a high incubation temperature is used, e.g., 55°C.

#### pH of the Medium

To select for acid-tolerant bacteria, a low-pH medium can be used. For example, to select for the lactobacilli present in cheddar cheese, the pH of the medium is maintained at 5.35 with an acetic acid/acetate buffer; other organisms in the cheese cannot grow well at such a low pH. Similarly, to select for alkali-tolerant organisms, a high-pH medium can be used. For example, to select for the cholera-causing bacterium, *Vibrio cholerae*, from a stool sample, we can use a medium with a pH of 8.5; most other intestinal bacteria are unable to grow at this pH.



## Cell Size and Motility

We can sometimes make use of a small cell diameter or of bacterial motility to achieve selection. For instance, *Treponema* species from the human oral cavity can be selected by taking advantage of both of these properties. A membrane filter having a pore size of  $0.15\ \mu\text{m}$  is placed on the surface of an agar plate and gingival scrapings are placed on the filter. The unusually small size of treponemes allows them to penetrate the pores of the filter to reach the underlying agar. Moreover, treponemes have the ability to swim through solid agar media; consequently, they migrate away from the filter and grow to form a hazy zone within the agar, from which they can be subcultured. Other bacteria from the oral cavity are either too large to penetrate the membrane filter or, if they can penetrate it, are unable to migrate away through the agar.

## Biological Methods of Selection

A disease-producing species occurring in a mixed culture can often be selected by taking advantage of its pathogenic properties. For example, a sputum sample containing *Streptococcus pneumoniae* is ordinarily contaminated by many other bacterial species. However, laboratory mice are extremely susceptible to infection by *S. pneumoniae*, and if the sputum sample is injected into a mouse the pathogen will multiply extensively. Nonpathogenic bacteria present in the sample will either be inhibited or killed by the defense mechanisms of the animal. In a sense, the animal serves as the selective medium.

How can the microbiologist know what selective media or conditions to use for a given species? Many selective methods are given in the references at the end of this chapter. Moreover, you can often devise a satisfactory selective procedure by comparing the characteristics of the species sought with those of the accompanying contaminants. Differences in these characteristics, e.g., in susceptibility to certain antibiotics, can provide the basis for a suitable selective procedure.

## Selection in Nature

It is important to realize that the principle of selection is not limited to the laboratory; it also commonly operates in nature. For instance, the occurrence of high salt concentrations in bodies of water such as the Dead Sea selects for extreme halophiles such as those of the genus *Halobacterium*. In lakes, the anaerobic, sulfide-containing zone on or above the sediment mud provides conditions that often favor the mass development of green or purple sulfide-oxidizing, phototrophic bacteria. The nodules that occur on the roots of leguminous plants contain bacteria of the genus *Rhizobium*, which are uniquely suited for nitrogen fixation in association with these plants. In many types of natural infections of humans or animals, a single, uncontaminated pathogenic bacterial species can often be obtained from a blood sample; blood from a healthy animal or human is normally free of bacteria. Numerous other examples of selective conditions in nature exist.

## PURE CULTURES

If the bacterial species being sought comprises a suitably high proportion of the mixed population, it can be isolated in pure culture. The descendants of a single isolation in pure culture comprise a **strain**. A strain is usually made up of a succession of cultures and is often derived from a single colony; however, the number of bacteria which gave rise to the original colony is usually unknown. If a strain is derived from a single parent cell, it is termed a **clone**. Each strain



is designated by an identifying number and its history is recorded (the source from which the isolation was made, the name of the person who made the isolation, the date of the isolation, and the culture collection in which the strain is maintained and from which it can be obtained for study).

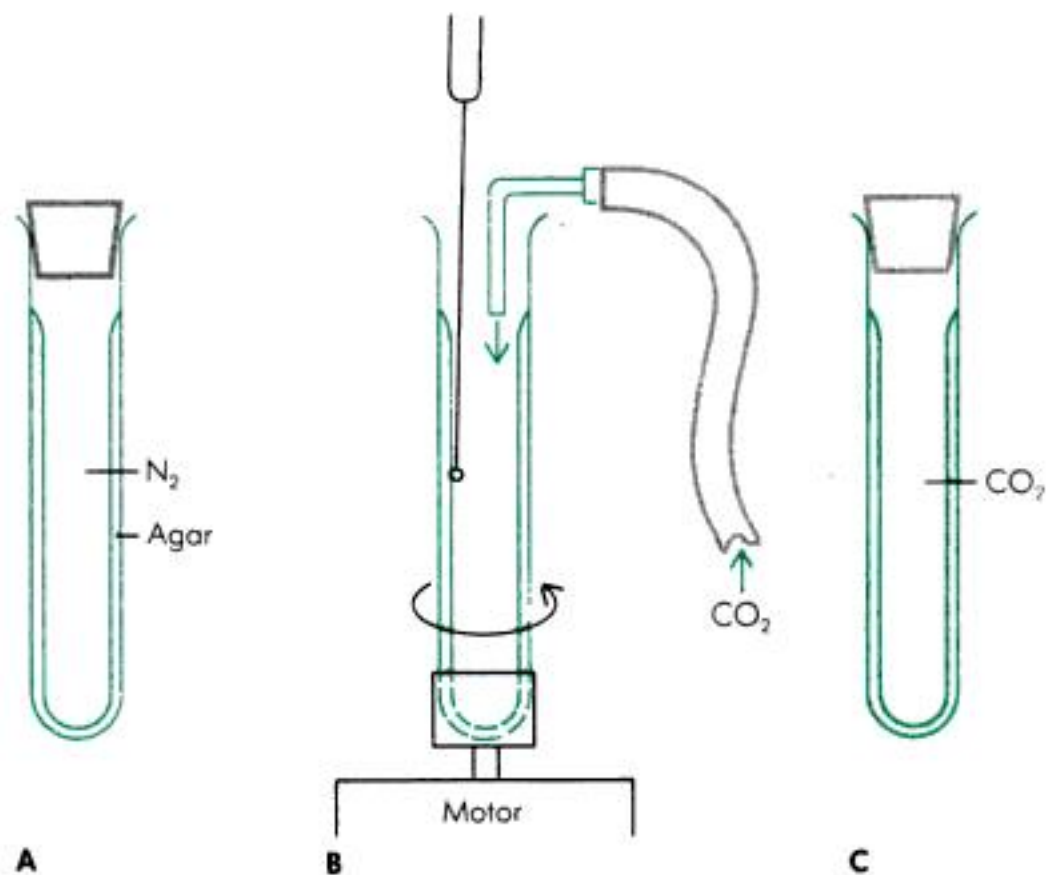
A variety of techniques have been developed whereby isolation into pure culture can be accomplished. Each technique has certain advantages and limitations, and there is no one method that can be used for all bacteria.

## Methods of Isolating Pure Cultures

### The Streak-Plate Technique

By means of a transfer loop, a portion of the mixed culture is placed on the surface of an agar medium and streaked across the surface. This manipulation "thins out" the bacteria on the agar surface so that some individual bacteria are separated from each other. Figure 8-2 illustrates a nutrient-agar plate culture that has been streaked to provide isolated colonies. When streaking is properly performed, the bacterial cells will be sufficiently far apart in some areas of the plate to ensure that the colony developing from one cell will not merge with that growing from another. Figure 8-3 illustrates a modification known as the **roll-tube technique** that is used for the isolation of stringent anaerobes.

**Figure 8-2.** Streak-plate culture showing areas of isolated colonial growth. Note that where the colonies are sparse they are larger than when crowded together. (Courtesy of Naval Biological Laboratory.)



**Figure 8-3.** Roll-tube method for isolating stringent anaerobes. (A) Stoppered anaerobic culture tube whose inner walls have been coated with a prereduced agar medium. The tube contains an atmosphere of oxygen-free  $N_2$ . (B) When the stopper is removed the tube is kept anaerobic by continuously flushing it with oxygen-free  $CO_2$  from a gas cannula. Inoculation is done with a transfer loop held against the agar surface as the tube is being rotated by a motor. By starting at the bottom and drawing the loop gradually upward, the inoculum becomes "thinned" to the point where well-isolated colonies can develop. (C) After inoculation the tube is restoppered and incubated.



The assumption is often made that a colony is derived from a single cell and, therefore, that the colony is a clone. However, this is not necessarily true. With species in which the cells form a characteristic grouping during cell division (for example, clumps of staphylococci or chains of streptococci), the colony may develop from a group of cells rather than from a single cell. Although not a clone, such a colony is nevertheless a pure culture if it contains only one kind of organism.

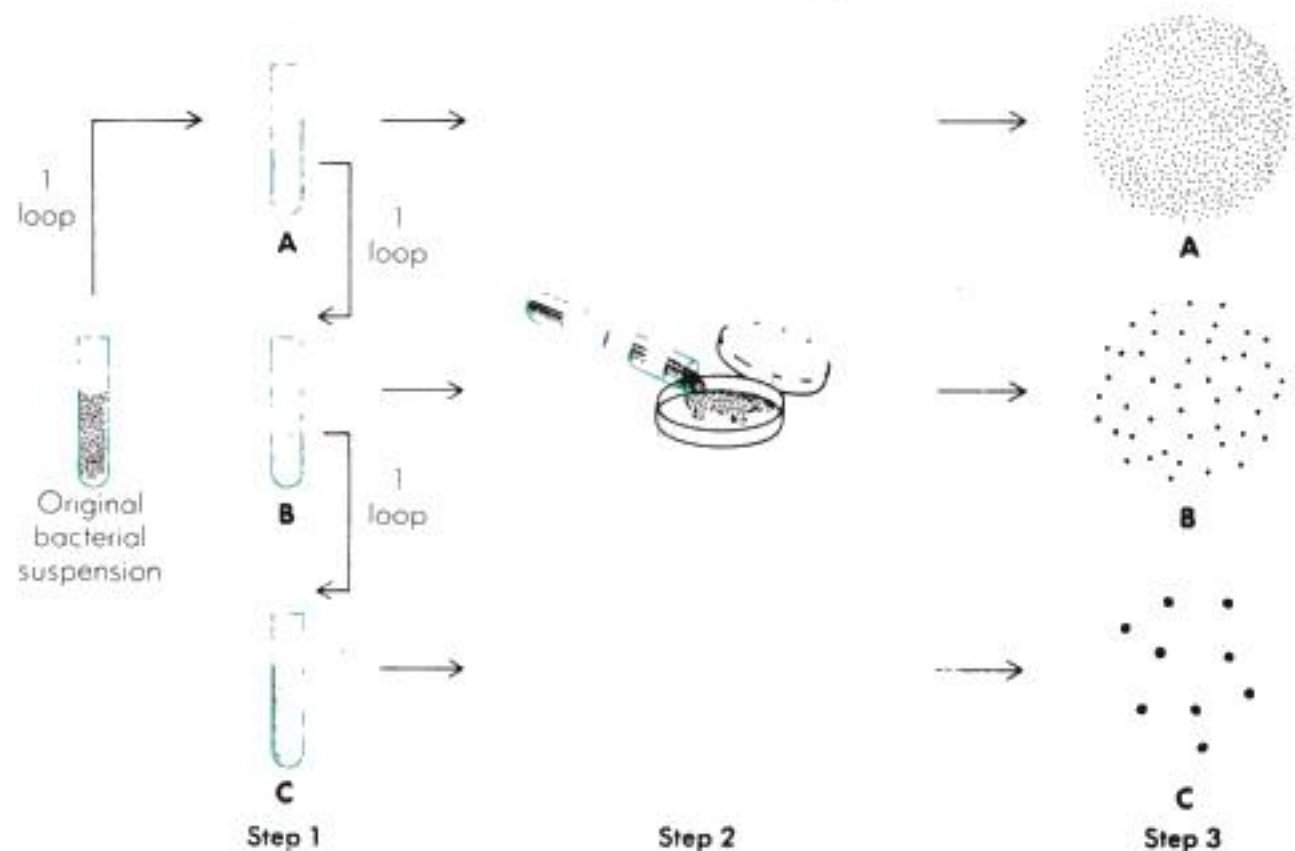
One should recognize that subculturing a colony from a single streak plate does not automatically assure purity. The colony may have been derived from two or more different kinds of bacteria. For example, when we attempt to isolate slime- or chain-producing bacteria, contaminants may be found to have adhered to the slime or to have been enmeshed in the network of chains, thereby resulting in impure colonies. The use of selective media can also lead to impure colonies. Although the growth of contaminants is inhibited on selective media, low numbers of viable cells may still be present, and such cells can be subcultured along with a colony. For these reasons, it is advisable to streak a culture several times in succession, preferably on nonselective media, in order to ensure purity.

### The Pour-Plate and Spread-Plate Techniques

**Figure 8-4.** Pour-plate technique is used for isolation of pure cultures of bacteria. Step 1: One loopful of original suspension is transferred to tube A (liquid, cooled agar medium). Tube A is rolled between the hands to effect thorough mixing of inoculum. Similar transfers are made from A to B to C. Step 2: Contents of each tube are poured into separate Petri dishes. Step 3: After incubation, plates are examined for the one which contains well-separated colonies. From this plate, pure cultures of bacteria can be isolated by transferring a portion of a colony to a tube of sterile medium.

In both of these methods the mixed culture is first diluted to provide only a few cells per milliliter before being used to inoculate media. Since the number of bacteria in the specimen is not known beforehand, a series of dilutions is made so that at least one of the dilutions will contain a suitably sparse concentration of cells.

In the **pour-plate method** the mixed culture is diluted directly in tubes of liquid (cooled) agar medium (see Fig. 8-4). The medium is maintained in a liquid state at a temperature of 45°C to allow thorough distribution of the inoculum. The inoculated medium is dispensed into Petri dishes, allowed to solidify, and then incubated. A series of agar plates showing decreasing numbers of colonies resulting from the dilution procedure in the pour-plate technique is shown in Fig. 8-4. The pour-plate technique has certain disadvantages. For







**Figure 8-5.** Spread plate showing colonies of two different bacterial species. A dilution of the mixed culture was spread over the surface with a glass rod. The large, dark colonies are *Serratia marcescens*, which has a brick-red pigment, and the smaller, light colonies are *Micrococcus luteus*, which has a lemon-yellow pigment. (Courtesy of Naval Biological Laboratory.)

instance, some of the organisms are trapped beneath the surface of the medium when it gels, and therefore both surface and subsurface colonies develop. The subsurface colonies can be transferred to fresh media only by first digging them out of the agar with a sterile instrument. Another disadvantage is that the organisms being isolated must be able to withstand temporary exposure to the 45°C temperature of the liquid agar medium; for instance, the pour-plate method would be unsuitable for isolating psychophilic bacteria.

In the **spread-plate method** the mixed culture is not diluted in the culture medium; instead it is diluted in a series of tubes containing a sterile liquid, usually water or physiological saline. A sample is removed from each tube, placed onto the surface of an agar plate, and spread evenly over the surface by means of a sterile, bent glass rod. On at least one plate of the series the bacteria will be in numbers sufficiently low as to allow the development of well-separated colonies (see Fig. 8-5). In contrast to the pour-plate technique, only surface colonies develop; moreover, the organisms are not required to withstand the temperature of liquid agar.

Unlike the streak-plate technique, the pour-plate and the spread-plate techniques may be performed in a quantitative manner to determine the number of bacteria (of a particular type) present in a specimen (see Chap. 7).

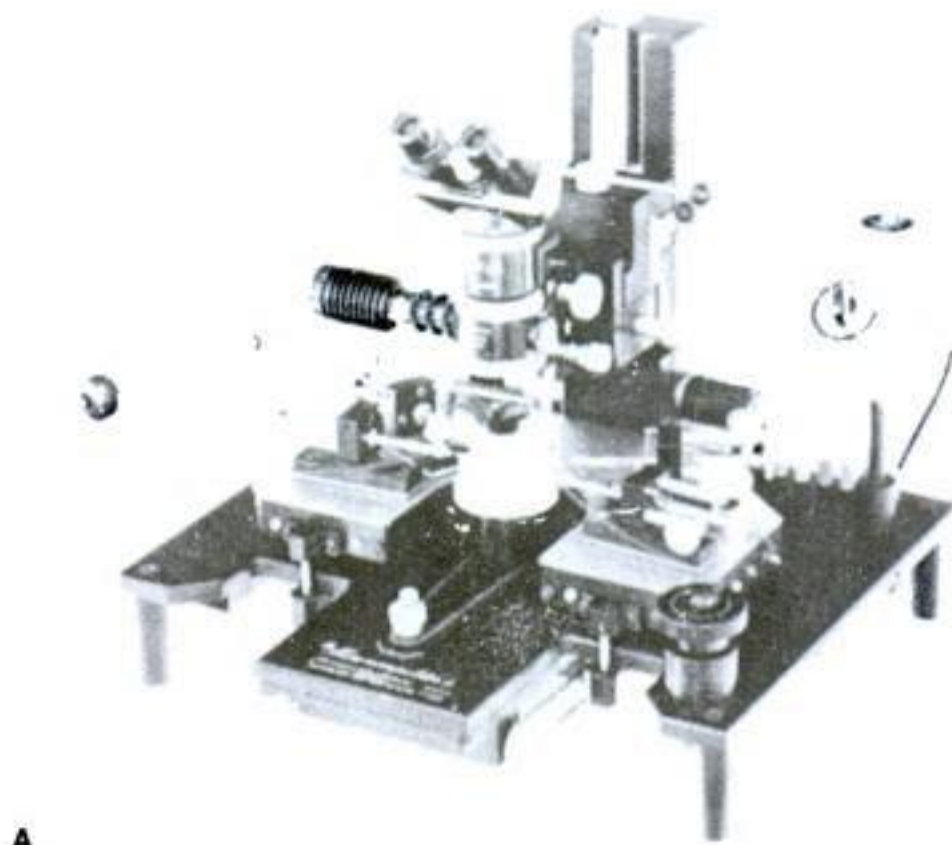
### Micromanipulator Techniques

A device called the **micromanipulator** can be used in conjunction with a microscope to pick a single bacterial cell from a mixed culture. The micromanipulator permits the operator to control the movements of a **micropipette** or a **microprobe** (a fine needle) so that a single cell can be isolated (see Fig. 8-6). This technique requires a skilled operator and is reserved for studies in which a clone must be obtained unequivocally.

### MAINTENANCE AND PRESERVATION OF PURE CULTURES

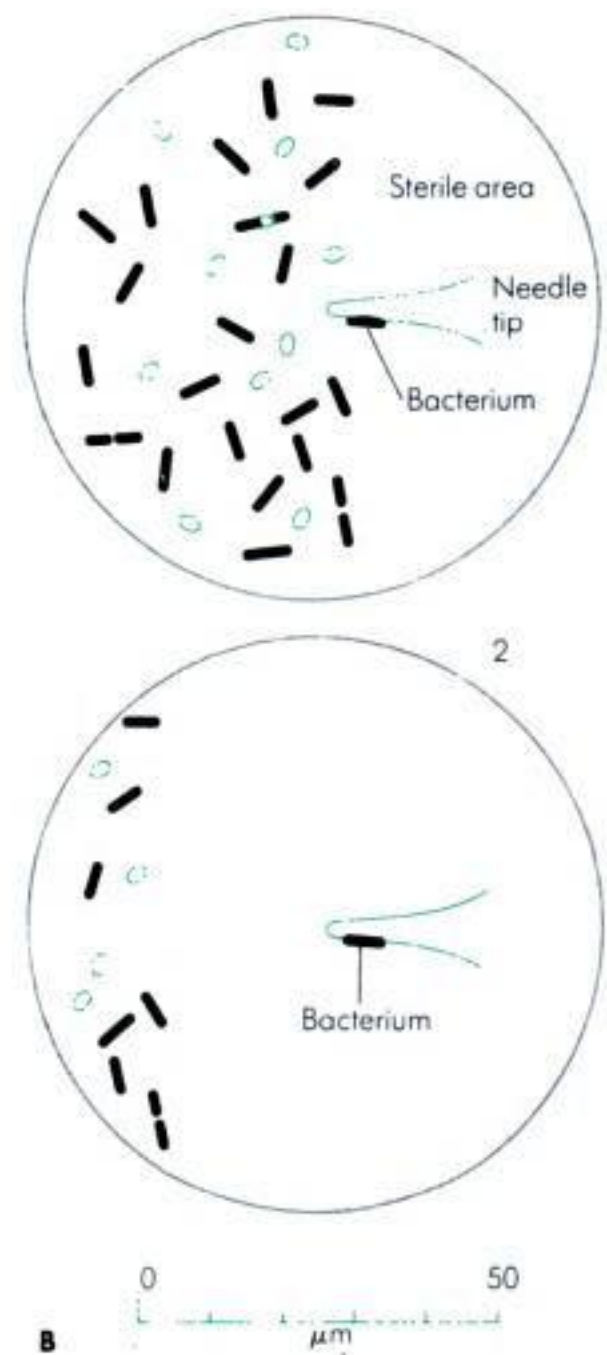
Most microbiology laboratories maintain a large collection of strains, frequently referred to as a **stock-culture collection**. These organisms are needed for laboratory classes and research work, as test agents for particular procedures, or as reference strains for taxonomic studies. Most major biological companies maintain large culture collections. The strains are used for screening of new, potentially effective chemotherapeutic agents; as assay tools for vitamins and amino





**A**

**Figure 8-6.** Isolation of single bacterial cells. (A) Micromanipulator equipment and microscope. The micromanipulator is equipped with probes that can position on objects a few  $\mu\text{m}$  in size. The manipulation of the probes is done while viewing the specimen through the microscope. (Micromanipulator Company.) (B) Schematic illustration showing isolation of a single bacterium from a mixture of cells on an agar gel. The microscope field (1) shows the point of the microprobe touching the agar gel near a bacterial cell, causing the organism to float in the small amount of water exuded from the gel. The microscope stage is then moved to the left (2) while the microprobe remains fixed; this causes the bacterium to follow the probe and become separated from the other cells. The agar block is subsequently dissected and the small piece containing the isolated bacterium is transferred to a sterile culture medium. (Courtesy of K. I. Johnstone, *Manipulation of Bacteria*, Churchill Livingstone, Edinburgh, 1973.)



**B**

acids; as agents for the production of vaccines, antisera, antitumor agents, enzymes, and organic chemicals; and as reference cultures that are cited in company patents. For these and other purposes it is extremely important to have properly identified and cataloged strains of bacteria available. Consequently, a considerable amount of research has been performed to develop methods whereby bacterial strains can be preserved and stored until they are needed. Several different methods have been developed, since not all bacteria respond in a similar manner to a specific method. Moreover, there are various practical considerations such as the amount of labor involved and the amount of storage space required. However, all the methods which we will now describe have the same objective: to maintain strains alive and uncontaminated and to prevent any change in their characteristics.

### Methods of Maintenance and Preservation

Strains can be maintained by periodically preparing a fresh stock culture from the previous stock culture. The culture medium, the storage temperature, and the time interval at which the transfers are made vary with the species and must



### Periodic Transfer to Fresh Media

be ascertained beforehand. The temperature and the type of medium chosen should support a slow rather than a rapid rate of growth so that the time interval between transfers can be as long as possible. Many of the more common heterotrophs remain viable for several weeks or months on a medium like nutrient agar. The transfer method has the disadvantage of failing to prevent changes in the characteristics of a strain due to the development of variants and mutants.

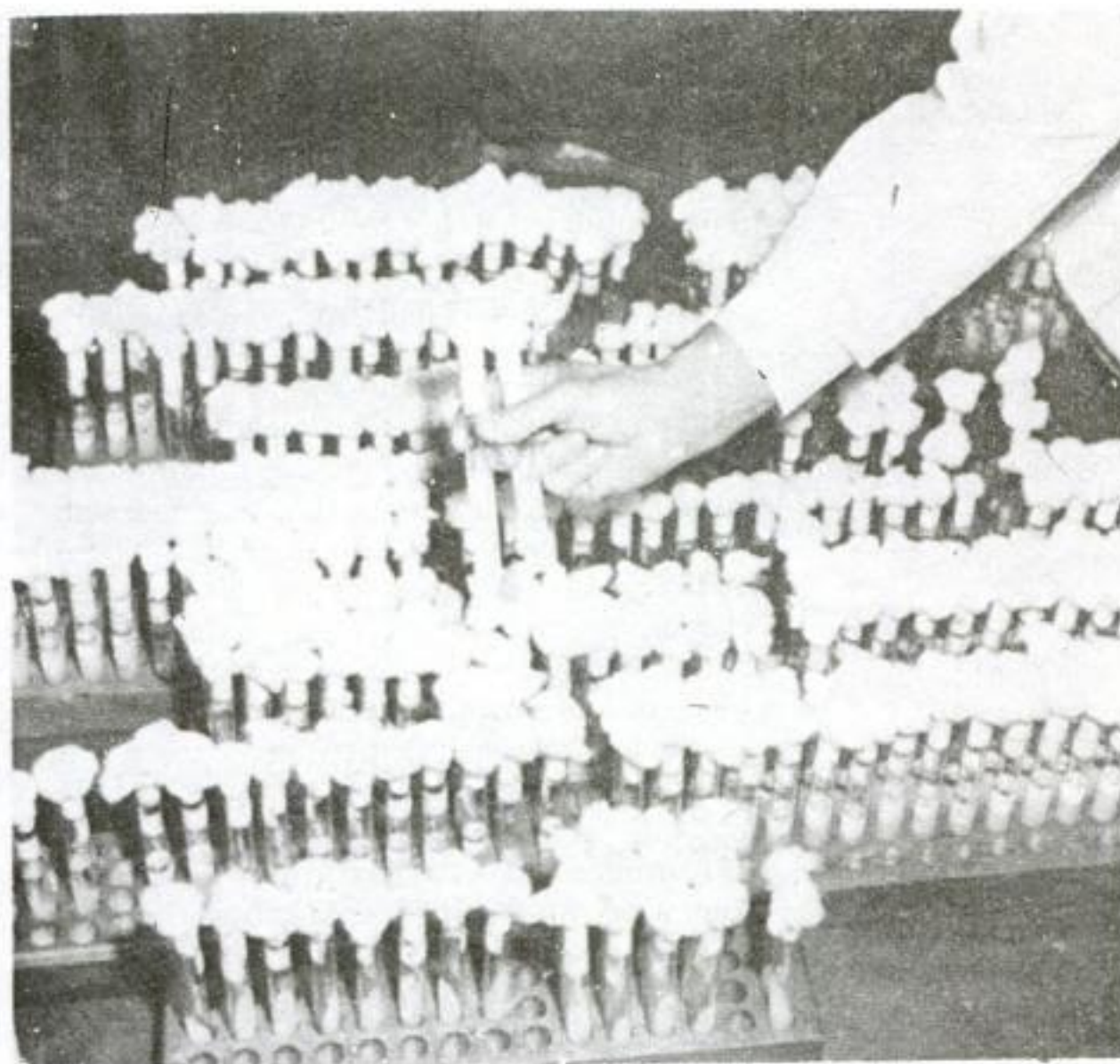
### Preservation by Overlaying Cultures with Mineral Oil

Many bacteria can be successfully preserved by covering the growth on an agar slant with sterile mineral oil. The oil must cover the slant completely; to ensure this, the oil should be about  $\frac{1}{2}$  in above the tip of the slanted surface. Maintenance of viability under this treatment varies with the species (1 month to 2 years). This method of maintenance has the unique advantage that you can remove some of the growth under the oil with a transfer needle, inoculate a fresh medium, and still preserve the original culture. The simplicity of the method makes it attractive, but changes in the characteristics of a strain can still occur. Figure 8-7 illustrates a culture collection maintained by this technique.

### Preservation by Lyophilization (Freeze-Drying)

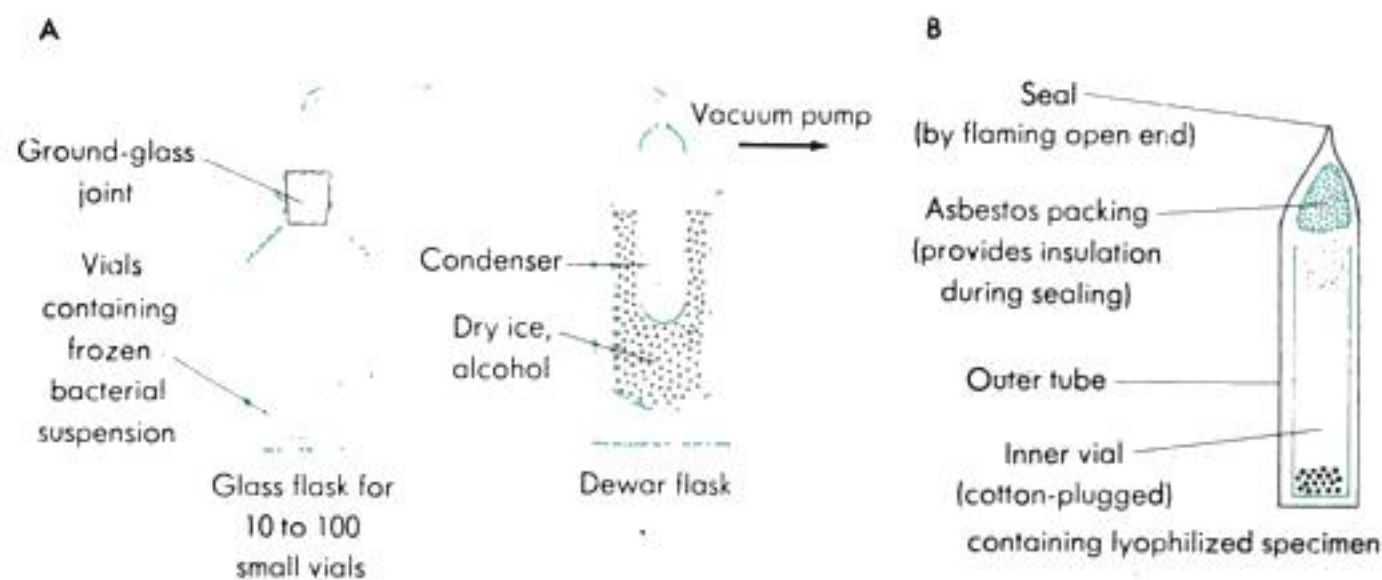
Most bacteria die if cultures are allowed to become dry, although spore- and cyst-formers can remain viable for many years. However, freeze-drying can satisfactorily preserve many kinds of bacteria that would be killed by ordinary

**Figure 8-7.** A culture collection maintained by overlaying cultures with mineral oil. (Courtesy of U.S. Department of Agriculture.)





**Figure 8-8.** Lyophilization process for preservation of cultures. (A) A simple apparatus for lyophilization. Small cotton-plugged vials containing frozen suspensions of bacteria are placed in the glass flask, which is attached to a condenser. The condenser is connected to a high-vacuum pump. The bacteria become desiccated as the ice in the frozen suspension sublimates directly to water vapor. The vapor is trapped on the cold surface of the condenser, thereby preventing it from entering the vacuum line and contaminating the pump oil. (B) After desiccation of the cultures as in (A), the vials are removed and each is placed in a larger tube. After insulating the vial with a plug of glass wool packing, the outer tube is hermetically sealed under a vacuum by means of torch. (Courtesy of American Type Culture Collection.)



drying. In this process a dense cell suspension is placed in small vials and frozen at  $-60$  to  $-78^{\circ}\text{C}$ . The vials are then connected to a high-vacuum line. The ice present in the frozen suspension sublimates under the vacuum, i.e., evaporates without first going through a liquid water phase. This results in dehydration of the bacteria with a minimum of damage to delicate cell structures. The vials are then sealed off under a vacuum and stored in a refrigerator. One arrangement of equipment employed to lyophilize cultures is shown in Fig. 8-8. Many species of bacteria preserved by this method have remained viable and unchanged in their characteristics for more than 30 years. Only minimal storage space is required; hundreds of lyophilized cultures can be stored in a small area. Furthermore, the small vials can be sent conveniently through the mail to other microbiology laboratories when packaged in special sealed mailing containers. Lyophilized cultures are revived by opening the vials, adding liquid medium, and transferring the rehydrated culture to a suitable growth medium.

### Storage at Low Temperatures

The ready availability of liquid nitrogen has provided the microbiologist with another very useful means for long-term preservation of cultures. In this procedure cells are prepared as a dense suspension in a medium containing a cryoprotective agent such as glycerol or dimethyl sulfoxide (DMSO), which prevents cell damage due to ice crystal formation during the subsequent steps. The cell suspension is sealed into small ampoules or vials and then frozen at a controlled rate to  $-150^{\circ}\text{C}$ . The ampoules or vials are then stored in a liquid nitrogen refrigerator (essentially a large tank having vacuum-insulated walls; see Fig. 8-9) either by immersion in the liquid nitrogen ( $-196^{\circ}\text{C}$ ) or by storage in the gas phase above the liquid nitrogen ( $-150^{\circ}\text{C}$ ). The liquid nitrogen method has been successful with many species that cannot be preserved by lyophilization, and most species can remain viable under these conditions for 10 to 30 years or more without undergoing change in their characteristics. However, the method is relatively expensive, since the liquid nitrogen in the refrigerators must be replenished at regular intervals to replace the loss due to evaporation.

### Culture Collections

When microbiologists first began to isolate pure cultures, each microbiologist kept a personal collection of those strains having special interest. Subcultures of some strains were often sent to other microbiologists; other subcultures were received and added to the scientist's own collection. Certain strains had tax-



onomic importance because they formed the basis for descriptions of species and genera. Others had special properties useful for various purposes. However, many important strains became lost or were inadequately maintained. Thus, it became imperative to establish large *central collections* whose main purpose would be the acquisition, preservation, and distribution of authentic cultures of living microorganisms.

Many countries have at least one *central collection*. As examples, in France a collection of bacteria is maintained at the Institut Pasteur in Paris; in England the National Collection of Type Cultures is in London; the Federal Republic of Germany maintains the Deutsche Sammlung von Mikroorganismen in Darmstadt; and Japan maintains a large collection at the Institute for Fermentation in Osaka. Many other such collections exist.

In the United States the major collection is the American Type Culture Collection (ATCC), located in Rockville, Maryland. In 1980 the collection included the following numbers of strains: bacteria, 11,500; bacteriophages, 300; fungi and fungal viruses, 13,700; protozoa, 720; algae, 130; animal-cell cultures, 500; animal viruses, rickettsiae, and chlamydiae, 1,135; and plant viruses, 220. More than 1 million ampoules of lyophilized or frozen living strains are inventoried and stored at the ATCC. Other large collections in the United States are more specialized in scope. For example, the Northern Utilization Research and Development Division, USDA, at Peoria, Illinois, maintains a collection of yeasts, molds, and bacteria especially for use in fermentations. The Quartermaster Research and Development Center, U.S. Army, Natick, Massachusetts, maintains a collection of microbial strains that are associated with deterioration processes. A number of smaller collections of a specialized nature also exist, such as the collection of anaerobic bacteria maintained by the Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg.

One of the major functions of a large national collection is the preservation of type strains. As discussed in Chap. 3, the type strain of a species has great taxonomic importance because it is the "name-bearer" strain, or permanent example, of the species. Microbiologists who propose a new species are expected to deposit the type strain with one or more national collections so that it can be preserved and so that subcultures can be distributed to other workers for study and comparison with other microorganisms.

**Figure 8-9.** (A) Liquid-nitrogen refrigerators used for preservation of bacteria. Each container holds many thousands of ampoules. (Courtesy of American Type Culture Collection.) (B) Preservation of bacterial cultures in the gas phase ( $-150^{\circ}\text{C}$ ) of liquid-nitrogen refrigerators. For preservation, a bacterial suspension is placed in a vial which is then sealed. This picture shows six vials, attached to a metal cane, being removed from storage. (Courtesy of Alma Dietz, The Upjohn Company.)





## CULTURAL CHARACTERISTICS

One of the major features of a bacterial strain is its appearance following growth on various media. Such commonplace characteristics as the abundance of the growth, the size of the colonies, and the color (or chromogenesis) of the colonies provide useful clues for identification.

To determine the growth characteristics of a bacterial strain, it is customary to observe the features of colonies and broth cultures. Inoculation of agar plates to obtain isolated colonies has already been described. Tubes of broth can be inoculated with the transfer needle or the loop; generally the loop is used when the inoculum is a liquid.

After inoculation of the medium and subsequent incubation, the cultural characteristics can be determined. The main features can be summarized as follows.

### Colony Characteristics

**Size.** Colonies range in size from extremely small (**pinpoint**), measuring only a fraction of a millimeter in diameter, to **large** colonies measuring 5 to 10 mm in diameter. Although the colonies of a given species have a characteristic diameter, one must be aware of certain factors affecting colony diameter. For instance, only well-separated colonies should be measured, since such colonies tend to have a larger diameter than those which are crowded together (for example, see Fig. 8-2). This is because widely separated colonies are subject to less competition for nutrients and less inhibition by toxic products of metabolism. Moreover, young colonies are smaller than older colonies; therefore the time at which measurements are made must be stated. There is generally an upper limit to the final size of the colonies of a given species; i.e., a point is reached where further incubation no longer results in a corresponding increase in size. However, some bacteria (e.g., certain species of *Proteus* and *Bacillus*) can spread across the entire agar surface, and the colony size is limited only by the dimensions of the Petri dish!

**Margin or Edge.** The periphery of bacterial colonies may take one of several different patterns, depending on the species. It may be evenly circular like the edge of a droplet or it may show irregularities such as rounded projections, notches, and threadlike or rootlike projections.

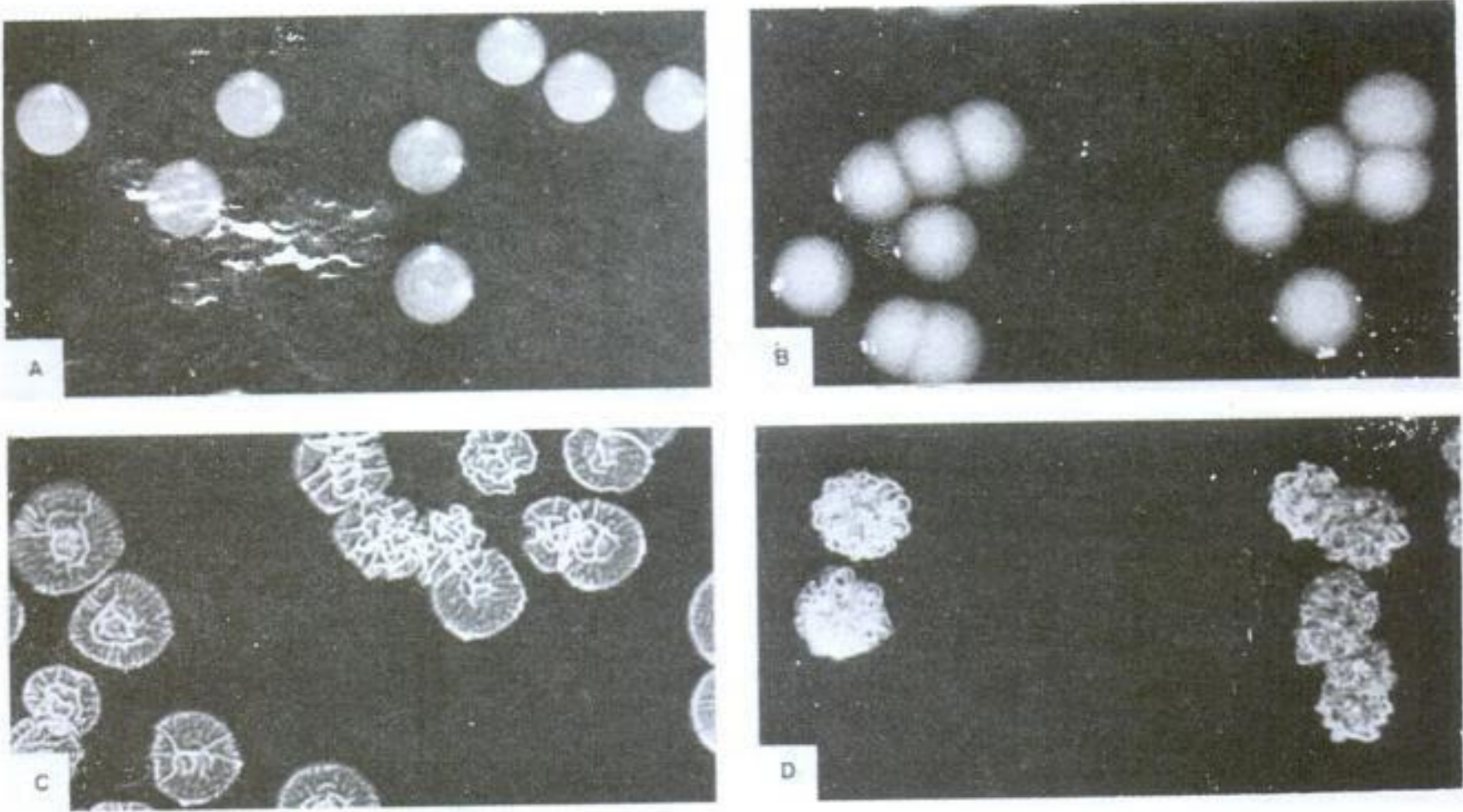
**Surface Texture.** Depending on the species, the colony surface may be **smooth** (shiny, glistening); **rough** (dull, granular, or matte); or **mucoid** (slimy or gummy). Certain species have colonies possessing a highly **wrinkled** surface.

For a pure culture, all the colonies on the plate should have a similar type of surface; however, you should bear in mind that some pure cultures may exhibit surface variation. One of the commonest variations is known as the **S — R variation**. This is due to the presence of mutant cells that give rise to some rough (R) colonies in a population that otherwise produces smooth (S) colonies. Some R mutants produce rough colonies because they lack the ability to make capsules, or, if the species is Gram-negative, they may no longer be able to form O antigens.

For several species of pathogenic bacteria, the surface texture of colonies may bear a relation to virulence. For instance, S colonies of *S. pneumoniae* or of *Salmonella* species are usually virulent, whereas R colonies are not. On the other hand, for strains of *Mycobacterium tuberculosis* a rough surface showing serpentine cords is usually a good indicator of virulence.



*image  
not  
available*



**Figure 8-10.** Bacterial colonies illustrating differences in characteristics. (A) Circular, raised, smooth surface; (B) circular, raised, finely granular surface; (C) irregular edge, flat, elevated folds in surface; (D) undulate edge, raised, irregularly elevated surface. (Courtesy of Naval Biological Laboratory.)

bacterial growth and thus deprive themselves of much useful information in the laboratory study of cultures.

To further emphasize the importance of cultural characteristics, suppose that we have prepared plate and broth cultures of an unidentified strain designated as strain 24. The colonies are irregular and raised and appear dry, with a roughened, granular surface. When we touch a colony with a transfer needle it proves to be brittle, and when a portion of the colony is removed it will not emulsify easily when spread in a drop of water. Growth in broth cultures occurs mainly in the form of a heavy surface pellicle, and the medium below the pellicle is only slightly turbid. Familiarity with the cultural appearance of bacteria would suggest that strain 24 might be an acid-fast bacterium (*Mycobacterium*). Additional tests must be performed to verify this possibility, but the cultural characteristics have provided a clue to the type of organism we are working with.

## QUESTIONS

- 1 Devise an enrichment procedure for an aerobic bacterial species that can use methane gas as a sole carbon and energy source.
- 2 During an epidemic of meningitis caused by *Neisseria meningitidis*, many people become healthy carriers (i.e., harbor the organism in their nasopharynx but do not have meningitis) and can spread the organism by coughing and sneezing. Suppose you are given the task of determining how many people in the epidemic region are healthy carriers. You soon discover that the human nasopharynx is inhabited by many different kinds of microorganisms. However, you learn from selected references that *N. meningitidis*



- is resistant to the chemotherapeutic agents vancomycin, colistin, and nystatin but is susceptible to penicillin. On the basis of this information devise a selective medium that could help you in your task.
- 3 What selective procedure would you use in the process of isolating from a soil sample (a) an endospore-forming organism, (b) a nonsporeforming gliding organism, (c) a psychrophilic organism?
  - 4 Give three examples of selective mechanisms occurring in nature that lead to the predominance of particular kinds of bacteria.
  - 5 Distinguish between the meanings of the terms mixed culture, pure culture, clone, and strain.
  - 6 Compare the advantages and disadvantages of the various techniques for the isolation of microorganisms in pure culture.
  - 7 What are the advantages and disadvantages of the various methods for preservation of pure cultures?
  - 8 Why have organizations been established to maintain pure cultures? Of what use are such collections?
  - 9 What difficulty might exist in subculturing the colony of a desired organism from a selective agar medium? What additional steps should be taken to help assure culture purity?
  - 10 How could you acquire a subculture of the type strain of *Streptococcus lactis* so that you could compare its characteristics with another strain that you have isolated from milk?
  - 11 What general categories of pigments are produced by bacteria? For each category give an example of an organism that makes such a pigment.
  - 12 Give several reasons why industrial biological companies maintain large stock-culture collections.

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# **PART THREE**

## **MICROBIAL PHYSIOLOGY AND GENETICS**



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## Chapter 9

# Enzymes and Their Regulation

- OUTLINE**
- Some Characteristics of Enzymes**
    - Chemical and Physical Properties of Enzymes**
    - Nomenclature of Enzymes**
    - How Enzymes are Named and Classified**
  - The Nature and Mechanism of Enzyme Action**
  - Conditions Affecting Enzyme Activity**
  - Inhibition of Enzyme Action**
  - Conditions Affecting Enzyme Formation**
  - Determination of Enzyme Activity**
  - Enzyme Preparations**
  - Regulation of Enzymes**
    - Mechanisms of Regulation of Enzymes**
    - Regulation of Enzyme Activity**
      - Energy-Link Control • Feedback Inhibition • Precursor Activation • General Processes Regulating Enzyme Activity
    - Regulation of Enzyme Synthesis**
      - Induction and Repression of Enzyme Synthesis • End-Product Repression • Catabolite Repression
  - Some Differences Between Procaryotic and Eucaryotic Enzyme Regulation**

A cell must be capable of performing a multitude of chemical changes in order to stay alive, grow, and reproduce. It may have to alter complex nutrients in the medium before they can enter the cell. It must effect additional changes on the nutrients once they are in the cell. These nutrients are broken down chemically to provide energy for the cell and simple precursors for the synthesis of cell material. The chemical changes involved are exceedingly complex, considering the diversity of materials used as foods on the one hand and the variety of substances synthesized into cell constituents on the other. How does the cell accomplish these changes? The answer lies in the activity of **enzymes**, substances present in the cell in minute amounts and capable of speeding up chemical reactions associated with life processes. Any impairment of enzyme activity is reflected by some change in the cell, or even by death. There can be no life without enzymes.



Within a living cell, enzymes function in sequences of reactions called **pathways**. For a cell to grow normally, it is essential that the flow of chemical substances, or **metabolites**, through these pathways be under a high degree of regulation or control. This regulation ensures that no products are deficient or in excess, and is exerted either on enzyme activity or on enzyme synthesis. Our purpose in this chapter is to describe some of the major characteristics of enzymes and how the enzymes are regulated.

## SOME CHARACTERISTICS OF ENZYMES

Certain substances in small amounts have the unique capacity of speeding up chemical reactions without themselves being altered after the reaction; they accelerate the velocity of the reaction without necessarily initiating it. Substances that behave in this manner are called **catalysts** or **catalytic agents**. For example, hydrogen and oxygen do not combine to any appreciable extent under normal atmospheric conditions. If, however, the two gases are allowed to touch colloidal platinum, they react instantaneously to produce water. The platinum greatly increases the speed at which this reaction takes place without being used up in the reaction. Nor do these substances ordinarily have any effect on the equilibrium of a reversible chemical reaction; they merely speed it up until it reaches equilibrium. They also exhibit specificity: a given substance will only affect a certain type of reaction. Enzymes are catalysts. However, unlike platinum, which is inorganic, enzymes are organic compounds produced by living organisms. Thus we may define an enzyme as an **organic catalyst** produced by a living cell. For example, in the cell the oxidation of a fatty acid to carbon dioxide and water takes place smoothly and rapidly within a narrow range of temperature and pH because of enzymes. Without these organic catalysts, as in a test tube, this same process requires extremes of pH, high temperatures, and corrosive chemicals.

Although all enzymes are initially produced in the cell, some are secreted through the cell wall and function in the cell's environment. Thus we recognize two types of enzymes on the basis of site of action: **intracellular enzymes**, or **endoenzymes** (functioning in the cell), and **extracellular enzymes**, or **exoenzymes** (functioning outside the cell). The principal function of the extracellular enzymes is to perform necessary changes on the nutrients in the medium to allow them to enter the cell. Intracellular enzymes synthesize cellular material

### The Origin of the Word *Enzyme*

The word *enzyme* was coined in 1878 by Kühne from a Greek term meaning "in yeast." Earlier, enzymes were referred to as "ferments" because their actions were similar to yeast fermentation. A major controversy on this subject occurred between Pasteur and the great German chemist Liebig. Liebig maintained that fermentations were caused by chemical substances not associated with living cells, but Pasteur held that the fermentation process was inseparable from living cells. As we know today, neither position was strictly wrong. The Pasteur-Liebig controversy was resolved in 1897 by Büchner, who demonstrated that a cell-free juice, prepared from yeasts by filtration, contained active enzymes.



and also perform catabolic reactions which provide the energy required by the cell.

The general characteristics of enzymes are the same whether the enzymes are produced by the cells of microbes, by people, or by other forms of life. In fact, cells from organisms that are very different may contain some enzymes that are identical or at least have identical functions.

In any one cell there are a thousand or more different enzymes. The enzymes present in a microbial cell are determined by the environmental conditions and by the cell's genetic constitution. This means that at any one moment, the enzyme content of a microbe is a reflection of the manner in which that cell copes with the environment.

## CHEMICAL AND PHYSICAL PROPERTIES OF ENZYMES

Enzymes are proteins or proteins combined with other chemical groups. Enzymes therefore possess the properties characteristic of proteins: they are denatured by heat, are precipitated by ethanol or high concentrations of inorganic salts like ammonium sulfate, and do not dialyze (pass through semipermeable membranes).

Many enzymes consist of a protein combined with a low-molecular-weight organic molecule called a coenzyme. The protein portion in this instance is referred to as the apoenzyme. When united, the two form the complete enzyme, identified as the holoenzyme, as shown below:

Apoenzyme	+	coenzyme	→	holoenzyme
Inactive		Inactive		Active
Protein		Organic molecule		
High molecular weight		Low molecular weight		
Nondialyzable		Dialyzable		

The integral part of some coenzymes is a vitamin. Several of the B vitamins, as listed in Table 9-1, have been identified as the main components of coenzymes.

A specific example of the relation of a vitamin to a coenzyme (vitamin B<sub>6</sub> in pyridoxal phosphate and pyridoxamine phosphate) is shown in Fig. 9-1.

In some instances the nonprotein portion of an enzyme may be a metal, e.g., iron in the enzyme catalase. The metal may be tightly bound to the protein or loosely bound and easily dissociable, depending on the specific enzyme. Many enzymes require the addition of metal ions (Mg<sup>2+</sup>, Mn<sup>2+</sup>, Fe<sup>2+</sup>, Zn<sup>2+</sup>, etc.) in order to be "activated." It is assumed that these metal ions function in combination with the enzyme protein, and they are regarded as inorganic coenzymes, or cofactors. Sometimes both a cofactor and a coenzyme (organic) are required before an enzyme becomes active.

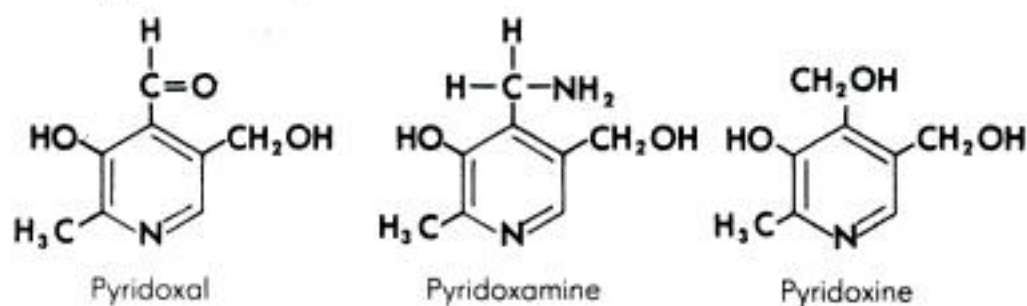
A large number of enzymes have been extracted from cells and, by a combi-

**Table 9-1.** Some vitamins and their coenzyme forms

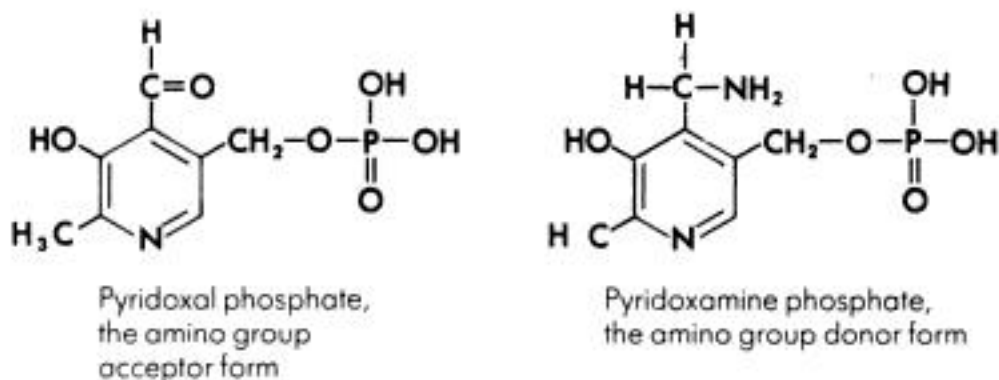
Vitamin	Coenzyme
Thiamine (B <sub>1</sub> )	Cocarcboxylase
Riboflavin (B <sub>2</sub> )	Riboflavin adenine dinucleotide
Niacin	Nicotinamide adenine dinucleotide
Pyridoxine (B <sub>6</sub> )	Pyridoxal phosphate
Folic acid	Tetrahydrofolic acid



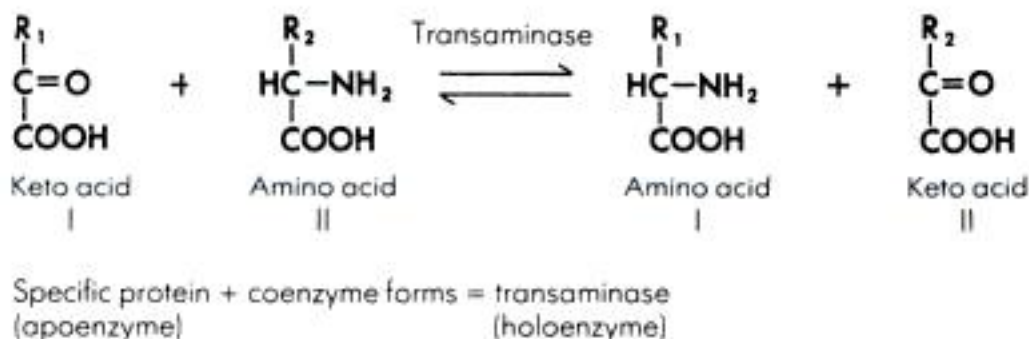
**A** Vitamin B<sub>6</sub> (three forms are pyridoxine, pyridoxal, and pyridoxamine)



**B** Coenzyme forms



**C** Transaminase reaction



**Figure 9-1.** (A) Vitamin B<sub>6</sub> exists in three forms. (B) Coenzyme forms of the vitamin. (C) The transaminase reaction.

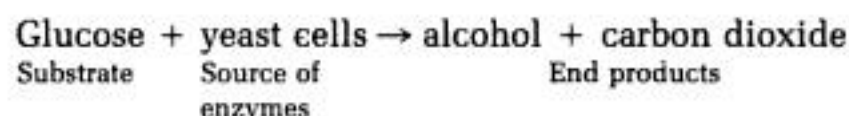
nation of physical and chemical techniques, have been obtained in chemically pure form. Urease was the first enzyme isolated in crystalline form; J. B. Sumner of Cornell University received the Nobel Prize in 1947 for this achievement. The protein nature of enzymes was accepted widely only after John Northrop and his colleagues, in the 1930s, crystallized pepsin and trypsin and found them also to be proteins. Enzymes are very large protein molecules; molecular weights from approximately 10,000 to 1 million have been determined for different enzymes. The enzyme catalase, which splits hydrogen peroxide into water and oxygen, has a molecular weight of 250,000. Hydrogen peroxide, on the other hand, has a molecular weight of 34.

Enzyme molecules are exceedingly efficient in accelerating the transformation of **substrate** (substance acted on by enzymes) to end product. A single enzyme molecule can effect the change of as many as 10,000 to 1 million molecules of substrate per minute. This ability, together with the fact that the enzyme is not consumed or altered in the reaction, reveals why very small quantities of enzymes are sufficient for cellular processes.

Enzymes are vulnerable to various environmental factors. Their activity may be significantly diminished or destroyed by a variety of physical or chemical

conditions, as will be shown later, but great differences exist among enzymes in this respect. Some may become inactivated by very minor alterations in the environment. The destruction of enzymes by physical or chemical agents results in a loss to the cell of the functions performed by the enzymes—a further revelation of their essential nature.

The two most striking characteristics of enzymes are (1) their high catalytic efficiency and (2) their high degree of specificity for substrates. One ounce of pure crystalline pepsin, for instance, can digest nearly two tons of egg white in only a few hours; whereas it would take 10 to 20 tons of strong acid 24 to 48 h at elevated temperatures without the enzyme. An enzyme molecule may transform  $10^2$  to  $10^6$  molecules of substrate per minute. A single enzyme may react with only a single substrate or, in some instances, with a particular chemical grouping on chemically related substrates. Essentially, this means that cells usually produce different enzymes for every compound they metabolize. Furthermore, each enzyme causes a one-step change in the substrate. For example, yeasts ferment glucose to alcohol and carbon dioxide. The initial reactants and the final products of the reaction are shown in the following equation:



This transformation is accomplished not by a single enzyme but by a group of enzymes, an enzyme system. More than a dozen single enzymes work in sequence, each performing a single specific change in the product formed by the preceding enzyme reaction. The last reaction in the system yields the final products. (Examples of enzyme systems will be found in Chap. 10.)

Today over 1,000 different enzymes are known, and well over 150 have been crystallized. Many more remain to be discovered. Exciting new areas of enzyme involvement have been discovered in recent years, including the self-regulating nature of many enzyme systems, the genetic control of enzyme function and synthesis, and the role of enzymes in development and differentiation. E.g., Thomas Cech and his colleagues at the University of Colorado reported in the journal *Cell* (31:147–157, 1982) the discovery of a biochemical reaction mediated by RNA in the absence of protein. The notion of catalytic RNA unfolds new perspectives on the basic components required for life, and also on the evolution of life. This finding has been described as “one of the most exciting discoveries of the decade.”

## NOMENCLATURE OF ENZYMES

### How Enzymes Are Named and Classified

In 1956 the International Union of Biochemistry established an international Commission on Enzymes to work on a systematic arrangement and nomenclature for the large and rapidly increasing number of enzymes. Its recommendations were first published in 1961, and they have been adapted and are universally used (see References). Briefly, these recommendations follow.

Except for a few of the originally studied enzymes such as pepsin, renin, and trypsin, most enzyme names end in *ase*, e.g., succinate dehydrogenase. When naming a complex of several enzymes on the basis of the overall reaction catalyzed by it, the word *system* should be used, e.g., the succinate oxidase



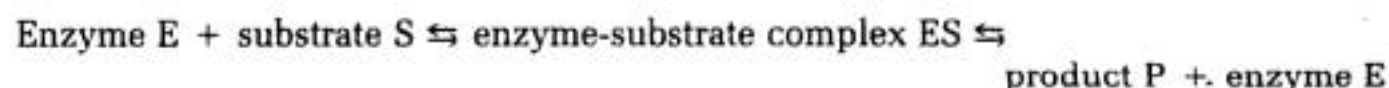
system, which catalyzes the oxidation of succinate by  $O_2$  and consists of succinate dehydrogenase, cytochrome oxidase, and several intermediate carriers. For classification, only single enzymes, and not enzyme systems, are considered.

The type of chemical reaction catalyzed is the basis for the classification and naming of enzymes, because it is this specific property that distinguishes one enzyme from another. Two names are recommended for each enzyme: a working or trivial name and a systematic name. The trivial name is shorter and more convenient to use; in many cases, it is the name already in current use. The systematic name is formed in accordance with definite rules, identifies the substrate or substrates, and specifies the type of reaction catalyzed. Furthermore, each enzyme is given an identifying classification number in addition to its trivial and systematic names. For example, E.C.1.1.1.1 is the Enzyme Commission number for alcohol dehydrogenase (trivial name); its systematic name is alcohol:NAD oxidoreductase. According to international classification, enzymes are grouped into six major classes (see Table 9-2). These groupings are based on the type of chemical reaction the enzymes catalyze.

Many enzymes occur in different structural forms but possess identical (or nearly so) catalytic properties. Such enzymes are called isozymes or isoenzymes. The present nomenclatural system makes no provision for structural diversity with similar catalytic function.

## THE NATURE AND MECHANISM OF ENZYME ACTION

Most enzyme reactions may be represented by the following overall reaction:



The enzyme E and substrate S combine to give an enzyme-substrate complex ES, which then breaks up to yield the product P. The enzyme is not used up in the reaction but is released for further reaction with another substrate molecule. This process may be repeated many times until all the available substrate molecules are consumed. However, equilibrium, a steady-state condition, is reached when the forward reaction rates equal the backward reaction rates. This is the basic equation upon which most enzymatic studies are based.

**Table 9-2.** Major classes of enzymes

Class No.	Class	Catalytic Reaction
1	Oxidoreductases	Electron-transfer reactions (transfer of electrons or hydrogen atoms)
2	Transferases	Transfer of functional groups (functional groups include phosphate, amino, methyl, etc.)
3	Hydrolases	Hydrolysis reactions (addition of a water molecule to break a chemical bond)
4	Lyases	Addition to double bonds in a molecule as well as nonhydrolytic removal of chemical groups
5	Isomerases	Isomerization reactions (reactions in which one compound is changed into an isomer, i.e., a compound having the same atoms but differing in molecular structure)
6	Ligases	Formation of bonds with cleavage or breakage of ATP (adenosine triphosphate)





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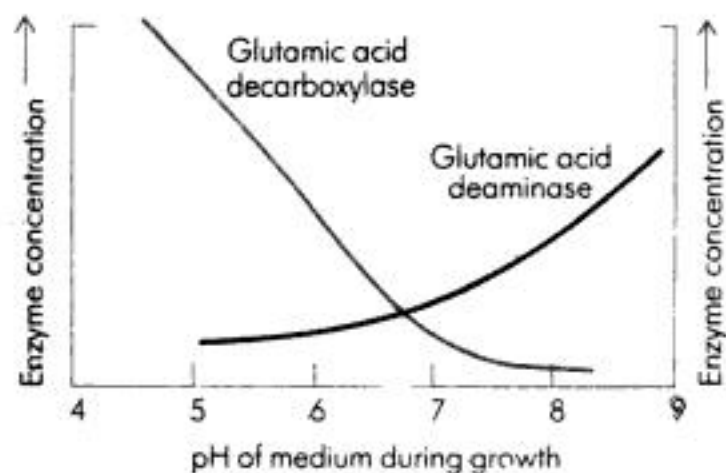


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**Figure 9-10.** Variations in the concentrations of glutamic acid decarboxylase and glutamic acid deaminase present in *Escherichia coli* with variations in the pH of the medium during growth. Note that at a low pH (acidic medium) glutamic acid decarboxylase predominates and at a high pH (basic medium), glutamic acid deaminase predominates.

ions, and form complexes with the metal. As already mentioned, many enzymes require a metal ion for their activity. Cyanide is a strong inhibitor of iron-containing enzymes because it "ties up" the iron, depriving the enzyme of an essential component. Similarly, fluoride inhibits enzymes which require calcium or magnesium by binding these metals. (It may be noted that the relatively small amount of fluoride which we find in tooth enamel as a result of water fluoridation does not exert this inhibitory action *in vivo*.) But the most important noncompetitive inhibitors are naturally occurring metabolic intermediates that can combine reversibly with specific sites on certain regulatory enzymes, thus changing the activity of their catalytic sites. This type of enzyme inhibition is referred to as noncompetitive inhibition since the inhibitor is not competing with the substrate for an active site on the enzyme surface and therefore is not reversed by increasing substrate concentration.

## CONDITIONS AFFECTING ENZYME FORMATION

The enzymatic content of animal-tissue cells is relatively constant, since they are in an environment which in terms of physical and chemical conditions is subjected to little change. But the bacterial cell is exposed to an ever-changing environment. For example, *Escherichia coli* may grow in an acid or alkaline medium (from pH 4.5 to pH 9.5), at room temperature or above body temperature, aerobically or anaerobically. Cells of *E. coli* grown at the extremes of these conditions do not contain the same kinds or amounts of enzymes (see Fig. 9-10). But the fact that the environment influences the formation of enzymes should not be misconstrued to mean that there is no consistent enzyme pattern for a given organism. Organisms manifest changes in reaction to environment only within certain limits. It is important to recognize their capacity for undergoing these changes when examining organisms. When organisms are studied physiologically, these studies must be performed under certain established conditions, which include the composition of the medium in which the cells are grown as well as the physical conditions during incubation.

## DETERMINATION OF ENZYME ACTIVITY

The enzymatic activity of bacteria (or of any cells or tissues) can be determined by a variety of techniques. Some procedures require special, elaborate instruments; others require only a test tube and a few reagents. All are based on a few simple principles. In order to carry out a quantitative assay of enzyme activity, it is necessary to know the following:

- 1 The nature of the reaction catalyzed
- 2 What cofactors and coenzymes are required
- 3 The required concentrations of both substrate and cofactor or coenzyme
- 4 The optimum pH
- 5 The optimum temperature
- 6 A simple analytical method for determining the disappearance of substrate or the appearance of products of the reaction

The substrate concentration should be above the saturation level so that the initial reaction rate is proportional to enzyme concentration alone. Coenzymes and cofactors should also be added in excess. Doing this ensures that the true limiting factor is the enzyme concentration (at its optimum pH and temperature). Generally, measurement of reaction product formation is more accurate than measurement of the disappearance of substrate. Such determinations of enzymatic activity have provided us with a wealth of knowledge concerning cells and their chemical reactions.

## ENZYME PREPARATIONS

The source of the enzymes used in any microbiological experiment may be one of the following: (1) cells in a growing culture, (2) cells removed from a growing culture and resuspended in a nonnutrient solution (resting cells, or nongrowing but viable cells), and (3) enzymes extracted from cells (cell-free enzyme preparation). Cell-free enzyme preparations may be of varying degrees of purity. They may be very crude extracts, as in the case when the material contains all the cellular contents recovered after fracturing the cell walls. However, this crude extract can be refined to obtain some particular fraction, e.g., ribosomal material, soluble cytoplasmic substance, or material of a certain molecular size range. In the last-mentioned instance, the objective may be to discover which subcellular entities house all the enzymes (enzyme system) responsible for certain cellular functions.

The manner in which each of these is used is as follows:

### GROWING-CULTURE TECHNIQUE

- 1 Inoculation of bacteria into medium containing substrate.
- 2 Incubation of bacteria (1 or more days of growth).
- 3 Examination for change or disappearance of substrate and presence of end products.

### RESTING-CELL TECHNIQUE

- 1 Growth of bacteria in a suitable medium.
- 2 Preparation of a resting-cell suspension. The cells are harvested from the medium by centrifugation and resuspended in a nonnutrient solution. The process is repeated to free the cells of all material from the medium and is known as **washing the cells**. The final suspension is referred to as a **resting-cell suspension**.
- 3 Addition of resting cells and substrate to special testing unit, e.g., Warburg apparatus or Thunberg tube.
- 4 Incubation.
- 5 Examination (or testing) for disappearance of substrate and appearance of end products.



## CELL-FREE ENZYME TECHNIQUE

- 1 Preparation of concentrated resting-cell suspension.
- 2 Disintegration of cells by special techniques to release enzymes, e.g., grinding, sonic treatment. (Any remaining whole cells are removed by centrifugation or filtration to obtain cell-free enzymes.)
- 3 Addition of cell-free enzymes and substrate to special testing unit.
- 4 Incubation.
- 5 Examination (or testing) for disappearance of substrate and appearance of end products.

The growing-culture technique is used routinely for the characterization of the enzymatic activities of microorganisms. Results of such tests provide information necessary for their identification. The resting-cell technique and cell-free enzyme preparations are principally used in research work where the object is to determine how the organism accomplishes each specific change. They provide favorable tools for the meticulous examination of the events that occur when a substrate undergoes change.

## REGULATION OF ENZYMES

A living cell contains upward of a thousand different enzymes, each of which is an effective catalyst for some chemical reaction. But these enzymes act together in a coordinated manner so that all the chemical activities in a living cell are integrated with one another. One consequence of this enzyme coordination is that the living cell synthesizes and degrades materials as required for normal growth and metabolism.

The control of cellular metabolism ultimately is accomplished by the regulation of enzymes. In a microbial cell, such as a bacterium, the existence of cellular regulatory mechanisms is all the more important because of the absence of supracellular controls, such as neural and hormonal controls, which are present in the tissue cells of higher organisms. Microorganisms have evolved a variety of enzyme regulatory mechanisms which accommodate the changing needs of the microbial cell in a changing environment.

## MECHANISMS OF REGULATION OF ENZYMES

Within the cell, there are two different regulatory mechanisms: the regulation of enzyme activity and the regulation of enzyme synthesis. Both mechanisms share the following properties:

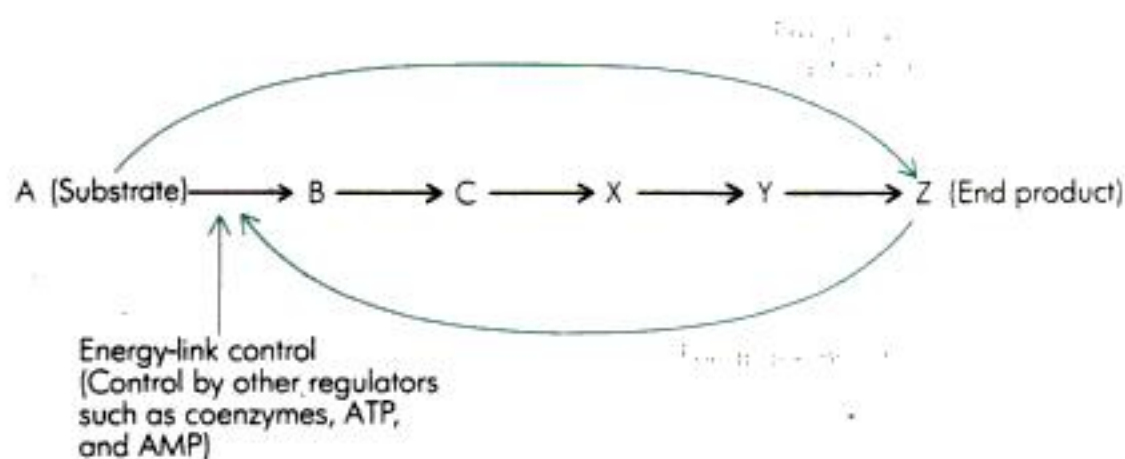
- 1 They are mediated or governed by low-molecular-weight compounds (molecules). These are either formed in the cell during metabolism or are found in the environment.
- 2 They involve the participation of a special class of control proteins. Such proteins are mediators of metabolic change as directed by the small effector molecules. There are generally two types of control proteins, namely, **allosteric enzymes** and **regulatory proteins**.

The activities of allosteric enzymes are enhanced or inhibited by combination with their effector molecules. (Allosteric enzymes are so called because the site on the enzyme molecule where an effector molecule acts is *different from the*



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**Figure 9-11.** Some mechanisms for the regulation of enzyme activity by direct control through a coupling of the catalytic mechanism with other processes. Feedback inhibition, precursor activation, and energy-link control are shown. See text for fuller explanation.

### General Processes Regulating Enzyme Activity

The cell can regulate enzyme activity by *less specific* or *more general* processes than those just described. These controls do not require the participation of effector molecules. The following cases illustrate this.

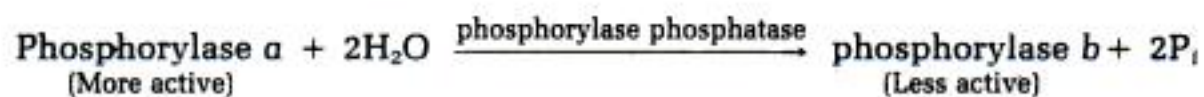
The cell membrane is a barrier to most hydrophilic molecules, but it has systems for the transport of specific compounds. Most of these systems require metabolic energy to function and therefore can be controlled by the availability of ATP.

The enzymatic reaction rate can be controlled by substrate concentration. As the substrate concentration increases, the reaction rate increases until a limiting value is reached when all the enzymes are saturated. And as the product accumulates, the reaction rate decreases. In addition, the concentrations of coenzymes and cofactors can exert controlling influences.

Control can also be effected by compartmentalization within the cell. Enzymes may be bound to various internal structures, especially membranes and macromolecules, so that enzymes and substrates are not in direct contact. The limiting physical access of enzymes to their substrates is more evident in the eucaryotic cell. Substrates can exist in separate **pools** because of their location within various membrane-bound organelles.

In some microbes, highly specific **proteolytic** (protein-degrading) **enzymes**, or **proteases**, break down other enzymes which are no longer required for metabolic reactions.

In a few instances, alteration in enzyme activity is brought about by a phenomenon called **covalent modification** of the regulatory enzyme molecule itself so that it can switch back and forth from an active to an inactive form. This modification is accomplished by the action of other enzymes. For example, the activity of phosphorylase *a* is increased by the hydrolytic action of phosphorylase phosphatase:



In turn, phosphorylase *b* can be changed back into the more active form, phosphorylase *a*, by another enzyme in the following manner:

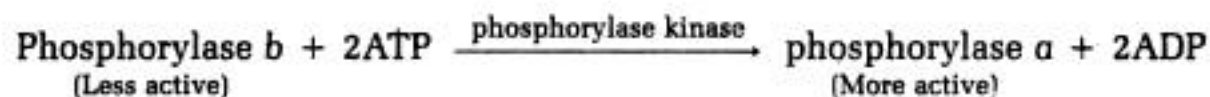


Figure 9-12 indicates the general processes involved in the regulation of enzyme activity.





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containing metabolites are also subject to catabolite repression if preferred nitrogen sources such as ammonium ion or glutamine are present. It is apparent that catabolite repression allows cells to save energy by not expending it on the synthesis of enzymes used in less efficient pathways.

### **SOME DIFFERENCES BETWEEN PROCARYOTIC AND EUCARYOTIC ENZYME REGULATION**

There are some aspects of enzyme regulation that are not the same between procaryotes and eucaryotes. For example, the difference between constitutive (basal) and fully induced enzyme levels is usually less for eucaryotes than it is for procaryotes. In the eucaryotic yeast *Saccharomyces cerevisiae* arginase is induced about 10-fold over the basal level while in *E. coli* the enzyme can be induced to about 100 times over basal level. In addition, in those eucaryotic organisms examined so far in sufficient genetic detail, there is no significant gene clustering into operons. Instead, the structural genes for enzymes in a specific biosynthetic pathway generally are scattered over many chromosomes and are not linked to each other. However, regulator genes can act at separate regulatory sites to coordinate enzyme synthesis.

### **QUESTIONS**

- 1 Why are enzymes important to a cell?
- 2 Define the following terms: apoenzyme, holoenzyme, coenzyme, cofactor, and pathway.
- 3 Describe two most striking characteristics of enzymes.
- 4 What is meant by an enzyme system?
- 5 Distinguish between a trivial name and a systematic name in enzyme nomenclature.
- 6 What are the six major classes of enzymes and their catalytic reactions?
- 7 What is the main function of an enzyme and how is it accomplished?
- 8 What is an active site of an enzyme?
- 9 Discuss the conditions that affect the activity of an enzyme.
- 10 Distinguish between the following types of inhibition of enzyme action. nonreversible, reversible, competitive, and noncompetitive inhibition.
- 11 Differentiate between constitutive and induced enzymes.
- 12 Describe the technique you would use to identify a microorganism by its enzymatic activities.
- 13 What advantages might possibly be gained by performing studies of enzyme action by means of resting cells in preference to a growing culture? Cell-free extracts in preference to whole cells?
- 14 Why is the existence of cellular regulatory mechanisms more important in a microbial cell than in a cell of a higher organism?
- 15 Describe the two different regulatory mechanisms of enzymes in a cell and the two general properties that they share.
- 16 What are allosteric enzymes?
- 17 Describe three specific types of regulation of enzyme activity.
- 18 Explain what is meant by the negative control of enzyme induction.
- 19 Give the meaning of the term operon.
- 20 What are the two types of feedback control in biosynthetic pathways?
- 21 How does the phenomenon of diauxy reflect the meaning of catabolite repression?



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- Lehninger, A. L.: *Principles of Biochemistry*, Worth Publishers, New York, 1982. This is a superb text in biochemistry written specifically for students taking their first course in biochemistry. It describes the biochemical aspects of cell structure and function with a molecular biology approach. Chapter 9 gives a clear, general treatment of the nature and characteristics of enzyme-catalyzed reactions.
- Mandelstam, J., and K. McQuillen (eds.): *Biochemistry of Bacterial Growth*, 2d ed., Blackwell Scientific Publications, Oxford, 1973. There is a good chapter on regulation entitled "Coordination of Metabolism." Many of the metabolic pathways depicted have arrows indicating modulator control. This innovation adds greatly to the reality that metabolic maps are intended to convey.
- Stanier, R. Y., E. A. Adelberg, and J. Ingraham: *The Microbial World*, 4th ed., Prentice-Hall, Englewood Cliffs, N.J., 1976. An advanced general text on microbiology with emphasis on the physiology and molecular biology of microorganisms. Chapter 8 is very relevant to enzyme regulation.

# Chapter 10

## Microbial Metabolism: Energy Production

### **OUTLINE**    **Some Principles of Bioenergetics**

#### **Oxidation-Reduction Reactions**

#### **The Respiratory Chain**

#### **Energy Production by Anaerobic Processes**

Glycolysis • The Pentose Phosphate Pathway • The Entner-Doudoroff Pathway • Fermentation

#### **Energy Production by Aerobic Processes**

The Tricarboxylic Acid Cycle • Energy Yield in Aerobic Respiration • Catabolism of Lipids • Catabolism of Proteins • Respiration without Oxygen in Some Bacteria • Heterotrophic CO<sub>2</sub> Fixation • The Glyoxylate Cycle

#### **Energy Production by Photosynthesis**

Cyclic and Noncyclic Photophosphorylation

#### **The Mechanism of ATP Synthesis**

The multiplicity of processes performed by all biological systems can be traced, directly or indirectly, to certain chemical reactions. Even the shape of a bacterium depends on such reactions: shape is determined by the geometric structure of the rigid peptidoglycan component of the cell wall, the peptidoglycan in turn being determined by the series of chemical reactions involved in its synthesis. The term **metabolism** denotes all the organized chemical activities performed by a cell, which comprise two general types, **energy production** and **energy utilization**. Energy is the ability to do work, and the work of a bacterial cell is extensive and varied. Energy is utilized for the construction of the physical parts of the cell such as wall or membrane; it is required for synthesis of enzymes, nucleic acids, polysaccharides, and other chemical components; it is required for repair of damage and mere maintenance of the status quo, as well as for growth and multiplication; it is required for accumulating certain nutrients in high concentration in the cell and for keeping certain other substances out of the cell; and it is required for motility. To support such extensive activities, vast amounts of energy must be provided. Under certain optimal conditions,



some bacteria have been found capable of metabolizing an amount of nutrient equivalent to their own weight every few seconds to provide such energy! A bacterial cell can be pictured as a dynamo of tremendous energy production. In this chapter, some basic principles of energetics will be discussed, followed by descriptions of just a few of the energy-producing mechanisms used by bacteria. Many of these mechanisms are also used by other microorganisms and by higher organisms, including human beings.

## SOME PRINCIPLES OF BIOENERGETICS

Most cells obtain energy by carrying out chemical reactions which liberate energy. Some cells are able to use light as a source of their energy, but even here the light energy must be converted into chemical energy to be in a form useful for the work of the cell.

In the course of any chemical reaction, energy available for the performance of useful work is either released or absorbed. The amount of energy liberated or taken up during the course of a reaction is referred to as the **free-energy change** ( $\Delta G$ ) of the reaction. Thus free-energy change can be defined as useful energy.  $\Delta G$  is expressed in terms of calories; however, this is merely a convenience since the free energy is not always in the form of heat but can, instead, be in the form of chemical energy. If the  $\Delta G$  of a chemical reaction has a negative value (such as  $-8000$  cal), the reaction releases energy (an **exergonic** reaction). If the  $\Delta G$  of a reaction has a positive value (such as  $+3000$  cal), the reaction requires energy (an **endergonic** reaction).

Concentration of reactants affects the value of  $\Delta G$  for a chemical reaction, and to make valid comparisons between the energetics of various reactions, a basis of reference must be used. For purposes of comparison, it is assumed that the concentration of all reactants is  $1.0$  M in the steady state; this is referred to as **standard concentration**.

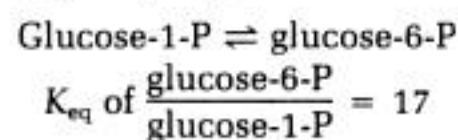
Under conditions of standard concentration, the free-energy change ( $\Delta G$ ) of a reaction is referred to by a special term,  $\Delta G^\circ$ . In other words,  $\Delta G^\circ$  is the amount of free energy released (or absorbed) when one mole of the reactant is converted to one mole of product at  $25^\circ\text{C}$  and one atmosphere of pressure, and, under (hypothetical) conditions, where all reactants and products are maintained at  $1$  M concentration.

The  $\Delta G^\circ$  or standard free-energy change is related to the equilibrium constant,  $K_{eq}$ , of a chemical reaction by the equation

$$\Delta G^\circ = -RT \ln K_{eq}$$

where  $R$  is the gas constant and  $T$  is the absolute temperature. If  $\Delta G^\circ$  is a negative value, the  $K_{eq}$  is greater than  $1.0$  and the formation of products is favored. If  $\Delta G^\circ$  is positive in value,  $K_{eq}$  is less than  $1.0$  and the chemical reaction tends to proceed in the reverse direction.

The value of  $\Delta G^\circ$  for a reaction can be calculated from the equilibrium constant of the reaction by using the above equation. For example, let us calculate  $\Delta G^\circ$  for the reaction catalyzed by phosphoglucumutase at  $25^\circ\text{C}$ :



The equation  $\Delta G^\circ = -RT \ln K_{eq}$  may be rewritten as:

$$\Delta G^\circ = -2.303RT \log K_{eq}$$

Substituting values for these terms, we get

$$\begin{aligned}\Delta G^\circ &= -2.303RT \log 17 \\ \Delta G^\circ &= -2.303(1.987)(298) \log 17 \\ \Delta G^\circ &= -1,680 \text{ cal/mole}\end{aligned}$$

Since the value of  $\Delta G^\circ$  is negative, the reaction can proceed from left to right under standard conditions.

Let us now calculate  $\Delta G$  under physiological conditions. ( $\Delta G$  is the actual free-energy change of a given chemical reaction under the conditions of concentration, pH, and temperature actually prevailing during the reaction, which are not necessarily the standard conditions as defined above.) Suppose that at 38°C the concentrations of glucose-6-P is  $1 \times 10^{-4}$  M and glucose-1-P are  $3 \times 10^{-5}$  M. We substitute these values in the following equation:

$$\Delta G = \Delta G^\circ + 2.303RT \log K_{eq}$$

$$\begin{aligned}\text{We get } \Delta G &= -1,680 + 2.303(1.987)(311) \log \frac{(1 \times 10^{-4})}{(3 \times 10^{-5})} \\ \Delta G &= -1,680 + 1,423 \log 3.3 \\ \Delta G &= -1,680 + 740, \\ \Delta G &= -940 \text{ cal/mole}\end{aligned}$$

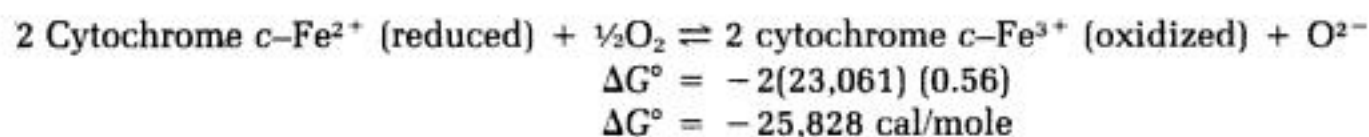
Thus, under physiological conditions, the reaction still proceeds from left to right.

It is also possible to obtain energy from a chemical reaction in the form of electric potential. Conversely, it is possible to use electric potential energy to drive a chemical reaction. Electric energy is generated when oxidations occur by the removal of electrons (as we will soon see). When the electrons fall through a potential difference or drop, energy is produced. The relationship between an oxidation-reduction potential difference and the standard free-energy change is

$$\Delta G^\circ = -nFE^\circ$$

where  $n$  is the number of moles of electrons transferred in the reaction,  $F$  is Faraday's constant (23,061 cal/V per equivalent), and  $E^\circ$  is the standard oxidation-reduction potential difference.

For example, let us calculate  $\Delta G^\circ$  for the reaction in which cytochrome  $c$  is oxidized by oxygen from the ferrous to the ferric state with  $E^\circ = +0.56$ V:

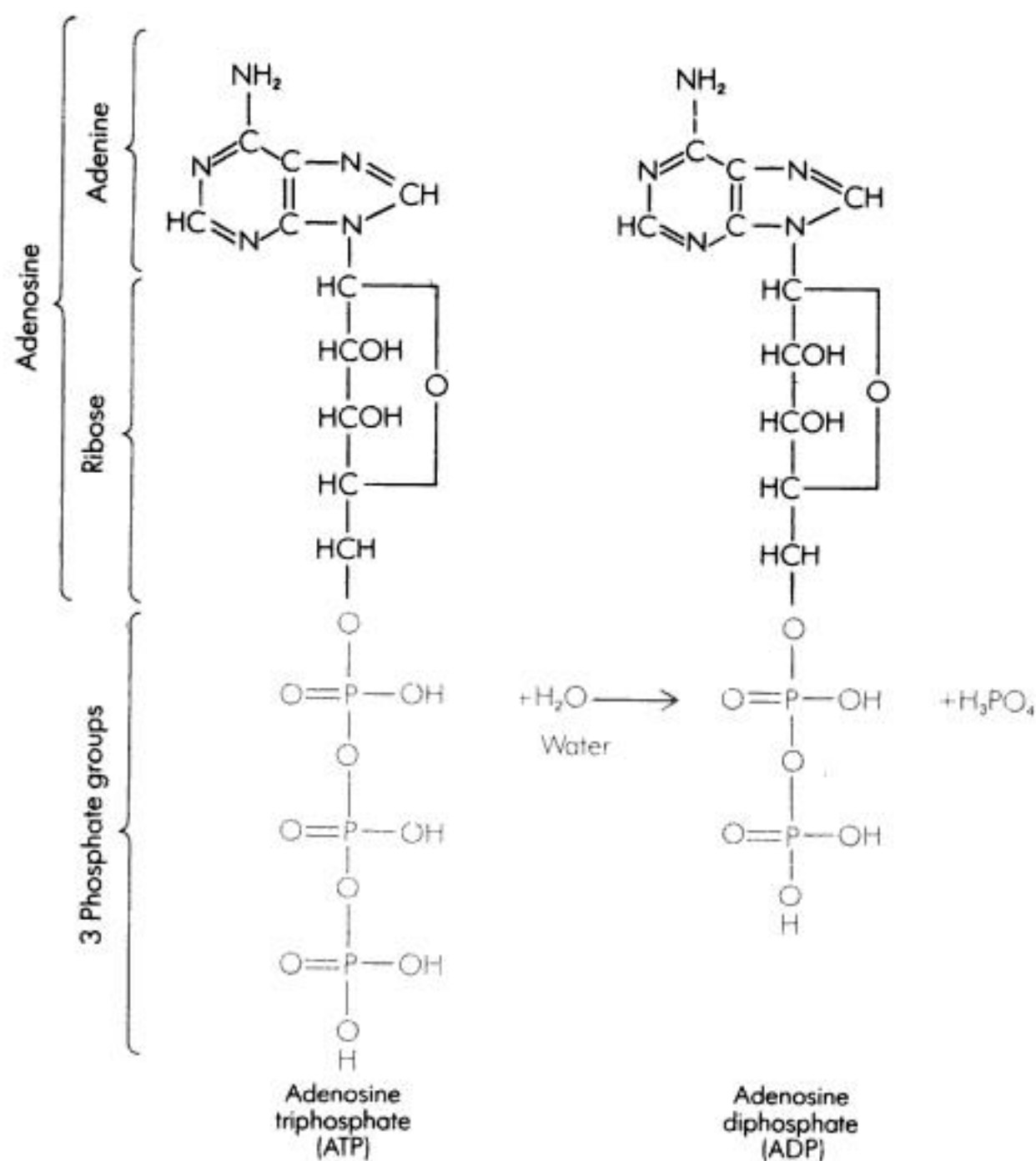


In order for life to continue, it is essential that the energy released from exergonic reactions be used to drive endergonic reactions, and living organisms have developed characteristic ways of coupling exergonic reactions with endergonic reactions. The basic principle involved is that there be a common reactant. This can be best understood by the following example.



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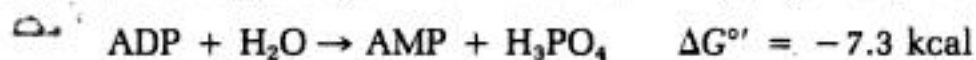


**Figure 10-1.** Hydrolysis of adenosine triphosphate.

Overall reaction:  
 $ATP + H_2O \rightarrow ADP + H_3PO_4; \Delta G^{\circ'} = -7.3 \text{ kcal mol}^{-1}$

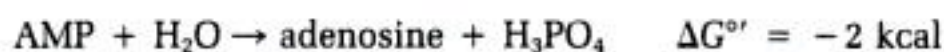
released' is a measure of the transfer energy capacity of ATP. It should be remembered, however, that the  $\Delta G^{\circ'}$  of  $-7.3 \text{ kcal}$  for ATP is not necessarily that existing in the intact cell. The pH and the concentrations of ATP, ADP,  $Mg^{2+}$  etc., in the cell are not identical to the standard conditions employed for determining  $\Delta G^{\circ'}$ . If appropriate corrections are made, the free energy of hydrolysis within the cell is closer to  $-12.5 \text{ kcal}$ , although this value can vary depending upon intracellular concentrations of various materials. However, for consistency and comparison, thermodynamic calculations of biological energy exchanges must be carried out under arbitrarily defined standard conditions.

The compound ADP (adenosine diphosphate) is also a high-energy-transfer compound, since its hydrolysis also liberates a large quantity of energy:



where AMP stands for adenosine monophosphate. AMP, however, is a low-

energy compound; its hydrolysis yields only a small quantity of energy:



Several types of chemical reactions are involved in energy production, but oxidation-reduction is probably the commonest. A discussion of some of the basic aspects of oxidation-reduction reactions will clarify their relationship to energy production.

## OXIDATION-REDUCTION REACTIONS

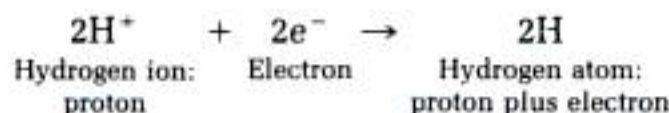
Oxidation is the **loss of electrons**; reduction is the **gain of electrons**. Frequently, oxidation reactions are **dehydrogenations** (reactions involving the loss of hydrogen atoms); since a hydrogen atom consists of a proton plus an electron, a compound which loses a hydrogen atom has essentially lost an electron and therefore has been oxidized.

An **oxidizing agent (oxidant)** will absorb electrons and will therefore become reduced, as illustrated by the following examples.

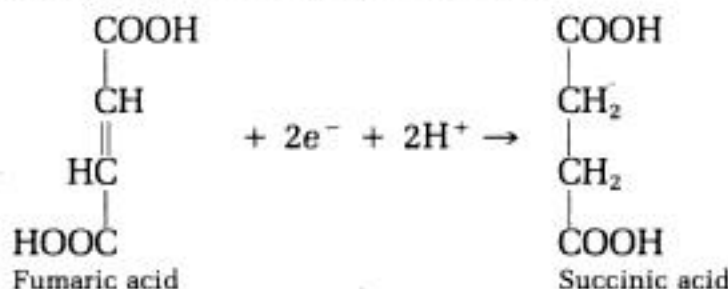
The ferric ion is an oxidizing agent; it absorbs electrons and becomes reduced to ferrous ion:



The hydrogen ion is an oxidizing agent; it absorbs electrons and becomes reduced to atomic hydrogen:



Fumaric acid is an oxidizing agent; it absorbs hydrogen atoms (which contain electrons) and becomes reduced to succinic acid:



A **reducing agent (reductant)** donates electrons, becoming oxidized in the process. The ferrous ion is a reducing agent, it donates electrons and becomes oxidized to ferric ion:



From this example, one can see that the reverse of each oxidation reaction is a reduction and the reverse of a reduction reaction is an oxidation. Moreover, in each reaction, a pair of substances is involved: one is the *reduced form*, the other the *oxidized form*, e.g., ferrous ion and ferric ion, succinic acid and fumaric acid. Each such pair of substances is referred to as an **oxidation-reduction (O/R) system**.

One O/R system may tend to absorb electrons from another O/R system; i.e., the first system will oxidize the second. On the other hand, the tendency of the



first system to absorb electrons may be so low that the second system may oxidize the first. This power (the tendency to absorb electrons) is expressed by the **standard oxidation-reduction potential** or the **electromotive potential** ( $E'_0$ ) of an O/R system, which is measured electrically under standardized conditions of comparison (electron donor and its conjugate at 1.0 M concentration, 25°C, and pH 7.0) and expressed in volts. The more positive the  $E'_0$ , the greater the oxidizing ability of the system. Consequently, any system listed in Table 10-2 can oxidize any other system listed above it, but not below it, under the standard conditions. Such relationships are very important in understanding the orderly sequence in which biological oxidations occur.

As discussed previously, when one O/R system oxidizes another, energy is released. It is important to know the values of  $E'_0$  for each system, because the  $\Delta G^\circ$  of the overall reaction is directly proportional to the difference in  $E'_0$  values. If the voltage difference is large, an amount of free energy sufficient to drive the synthesis of ATP may be liberated.

In respiration, an oxidizable substrate is the primary electron donor. In **aerobic** respiration the terminal electron acceptor is oxygen; in **anaerobic** respiration the final electron acceptor is a compound like fumarate,  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$ , or  $\text{CO}_3^{2-}$ . In fermentation, an organic compound is the final electron acceptor; an oxidizable substrate is the electron donor. In photosynthesis carried out by bacteria, bacteriochlorophylls serve as both electron donors and acceptors. In photosynthesis by green plants, algae, and cyanobacteria, water serves as a primary electron donor and  $\text{NADP}^+$  (nicotinamide adenine dinucleotide phosphate) as a terminal electron acceptor. The paths through which these electrons flow in the various processes are called electron-transport chains.

**Electron-transport chains** are sequences of oxidation-reduction reactions that occur in cells. These reactions are mediated by a number of electron carriers and electron-carrier enzymes (discussed later). As the electrons flow through the chains, much of their free energy is conserved in the form of ATP; this process is called **oxidative phosphorylation**.

The multicomponent electron-transport chains are always associated with membranes. In eucaryotes, they are in mitochondrial or chloroplast membranes; in procaryotes, they are in the cytoplasmic membrane.

## THE RESPIRATORY CHAIN

A respiratory chain is an electron-transport chain. When a pair of electrons or hydrogen atoms (which contain electrons) from an oxidizable substrate is coupled with the reduction of an ultimate electron acceptor, such as oxygen, there is a large free-energy change ( $\Delta G^\circ$ ). The flow of electrons through the transport chain allows a stepwise release of this energy, some of which is conserved in the form of ATP at several steps in the chain. At these specific steps the difference in  $E'_0$  values is great enough to permit sufficient energy to be liberated for oxidative phosphorylation to occur.

The component O/R systems of a common type of respiratory chain are shown in Table 10-2 and are illustrated in Fig. 10-2.

A respiratory chain consists of enzymes having prosthetic groups or coenzymes. These can be regarded as the working parts of the enzymes, and in the case of the respiratory chain each is in fact an O/R system. The oxidized form of each prosthetic group or coenzyme has an absorption spectrum different from





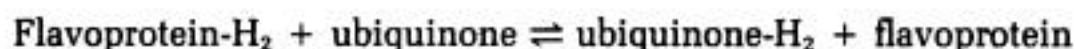
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reduced form:  $\text{Riboflavin} + 2\text{H} \rightleftharpoons \text{riboflavin-H}_2$

The reduced forms of the coenzymes are  $\text{FADH}_2$  and  $\text{FMNH}_2$ .

### Coenzyme Q

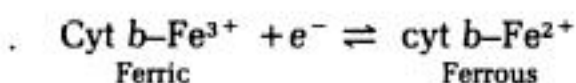
Coenzyme Q is also called **ubiquinone** because it is a quinone and is present in all cells. Coenzyme Q is a fat-soluble coenzyme. It functions as an acceptor of reducing power from the flavin-linked dehydrogenases:



$\text{NAD}^+$ ,  $\text{NADP}^+$ , flavoproteins, and ubiquinones carry  $2\text{H}^+$  and  $2\text{e}^-$ , but the cytochromes (discussed below) transfer only electrons, the protons being associated with an  $-\text{NH}_2$  group or a  $-\text{COO}^-$  group and eventually transferred to  $\text{O}_2$  (see Fig. 10-2).

### Cytochromes

Another major class of oxidative enzymes in the respiratory chain is the **cytochromes**. The prosthetic group of a cytochrome is a derivative of **heme** and contains a single iron atom, which is responsible for the oxidative or reductive properties of the enzyme. On the basis of differences in absorption spectra, cytochromes can be divided into three main categories: cytochromes *a*, cytochromes *b*, and cytochromes *c*. Each of these groups has a different function in the respiratory chain and can be further subdivided on the basis of minor differences in absorption spectra, e.g., cytochromes *c* and *c*<sub>1</sub> or cytochromes *a* and *a*<sub>3</sub>. Each cytochrome type can exist in either an oxidized or reduced form, depending on the state of the iron atom contained in their structure:



The cytochromes act sequentially to transport electrons from coenzyme Q to  $\text{O}_2$ . Cytochromes *a* and *a*<sub>3</sub> together are called **cytochrome oxidase**. Both of them also contain copper. But only cytochrome *a*<sub>3</sub> can react directly with oxygen.

### Sequence of Oxidation

The arrangement of O/R systems in Table 10-2 according to  $E'_0$  values is based on the experimentally determined sequence of oxidation reactions in the respiratory chain illustrated in Fig. 10-2. Sufficient energy for ATP synthesis is liberated at three points along the chain. The incremental release of energy in the respiratory chain results in a more efficient trapping of energy in ATP than would be true of direct oxidation of the reduced substrate by oxygen.

The respiratory chain of bacteria is associated with the cytoplasmic membrane; that of eucaryotes is present in mitochondrial membranes. Much of the electron transfer in membranes is accomplished within highly integrated particles or complexes.

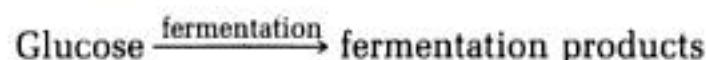
## ENERGY PRODUCTION BY ANAEROBIC PROCESSES

Heterotrophic bacteria can use a variety of organic compounds as energy sources. These compounds include carbohydrates, organic and fatty acids, and amino acids. For many microorganisms the preferred compounds are carbohydrates, especially the 6-carbon sugar glucose.



## Glycolysis

The most common pathway of glucose catabolism is the Embden-Meyerhof pathway of **glycolysis** ("splitting of sugar"). This process occurs very widely and is found in microorganisms as well as in animals and plants. Glycolysis does not require the presence of oxygen and therefore can occur in both aerobic and anaerobic cells. Aerobic cells degrade glucose by glycolysis, and this process constitutes the preparatory stage for the aerobic phase of glucose oxidation. Thus, under anaerobic conditions this situation prevails:



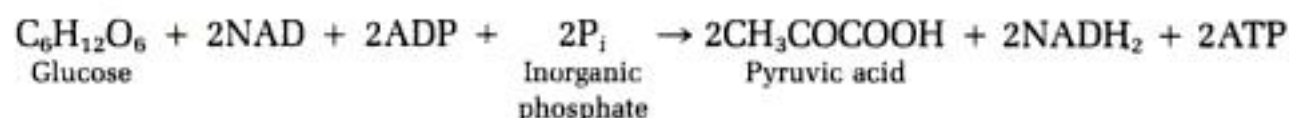
whereas under aerobic conditions, the following occurs:



In glycolysis, as shown in Fig. 10-3, fructose-1,6-diphosphate formed from glucose is split into two 3-carbon units (dihydroxyacetone phosphate and glyceraldehyde-3-phosphate), and they are subsequently oxidized to pyruvic acid. At the step where glyceraldehyde-3-phosphate is oxidized, a pair of electrons (two hydrogen atoms) is removed. In the absence of oxygen, this pair of electrons may be used to reduce pyruvic acid to lactic acid or ethanol. In the presence of oxygen, this pair of electrons may enter the respiratory chain.

Many of the reactions of the glycolytic pathway are freely reversible and can be used for the synthesis of glucose as well as for its breakdown. Only three of the reactions are not reversible by common enzymes; but the presence of other enzymes can reverse them for glucose synthesis to occur. Thus phosphoenolpyruvate is synthesized from pyruvate by the action of phosphoenolpyruvate synthase and specific phosphatases hydrolyze fructose-1,6-diphosphate and glucose-6-phosphate in the biosynthetic direction. The enzymes at these steps in the degradative direction are kinases and require ATP. (See Fig. 10-3.) For each molecule of glucose metabolized, two molecules of ATP are used up and four molecules of ATP are formed. Therefore for each molecule of glucose metabolized by glycolysis, there is a net yield of two ATP molecules. This is shown in Fig. 10-3.

The overall reaction of glycolysis can be summarized as follows:

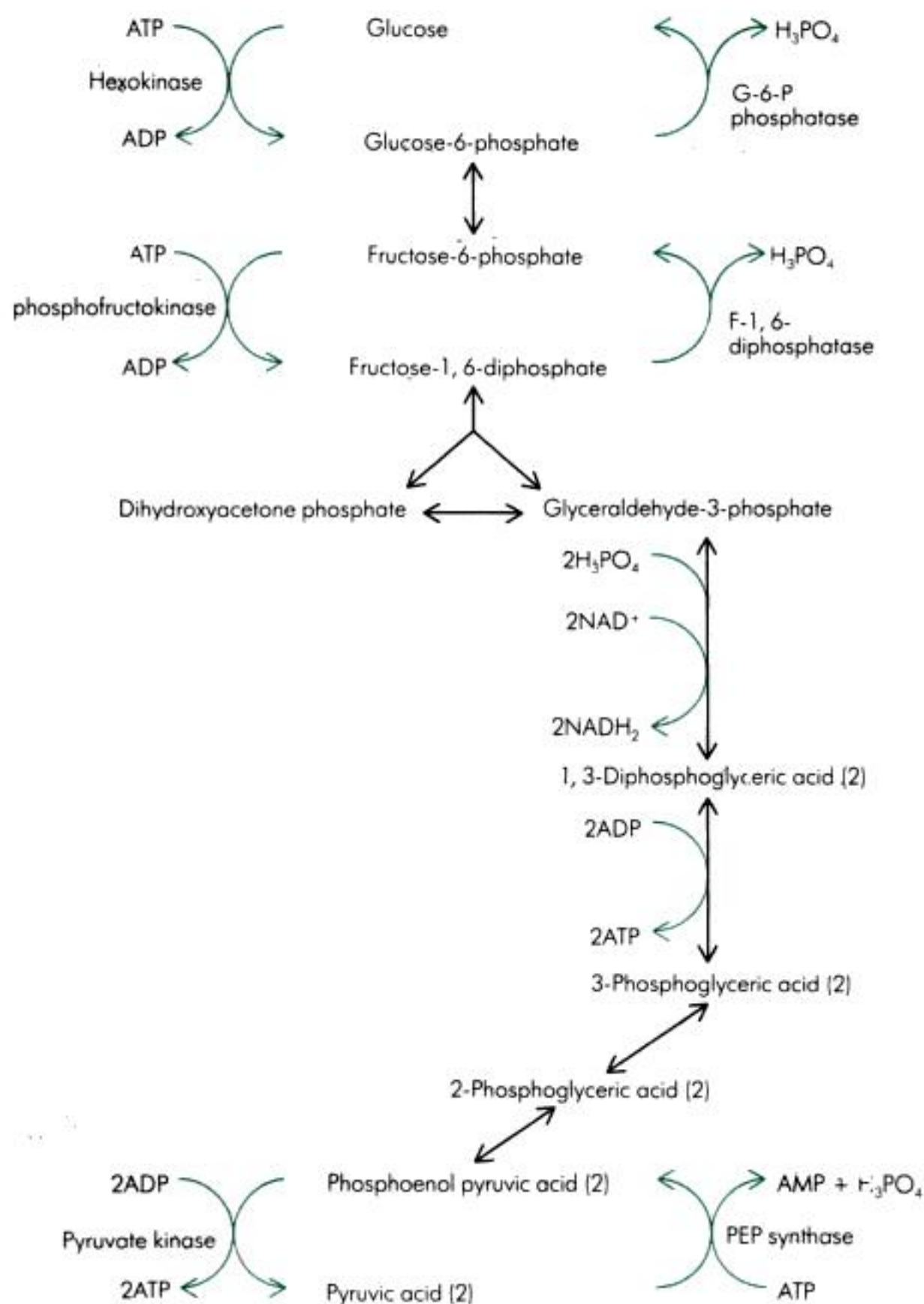


## The Pentose Phosphate Pathway

The pentose phosphate pathway, like the glycolytic one, is another catabolic reaction pathway that exists in both procaryotic and eucaryotic cells. Since it involves some reactions of the glycolytic pathway, it has been viewed as a "shunt" of glycolysis; hence it may also be called the hexose monophosphate shunt. Its other synonym is the phosphogluconate pathway.

Glucose can be oxidized by the pentose phosphate pathway with the liberation of electron pairs, which may enter the respiratory chain. However, this cycle is not generally considered a major energy-yielding pathway in most microorganisms. It provides reducing power in the form of  $\text{NADPH} + \text{H}^+$ , which is required in many biosynthetic reactions of the cell, and it provides pentose phosphates





**Figure 10-3.** The Embden-Meyerhof (glycolytic) pathway of glucose catabolism. Enzymes shown are for those steps which are not freely reversible by a common enzyme.

for use in nucleotide synthesis. Although it can produce energy for the cell as an alternate pathway for the oxidation of glucose, it is also a mechanism for obtaining energy from 5-carbon sugars.

As seen in Fig. 10-4, the pentose phosphate pathway involves the initial phosphorylation of glucose to form glucose-6-phosphate; the latter is oxidized to 6-phosphogluconic acid with the simultaneous production of NADPH. Decarboxylation of 6-phosphogluconic acid, together with a yield of NADPH, produces ribulose-6-phosphate. Epimerization reactions yield xylulose-5-phosphate and ribose-5-phosphate. These two compounds are the starting point for



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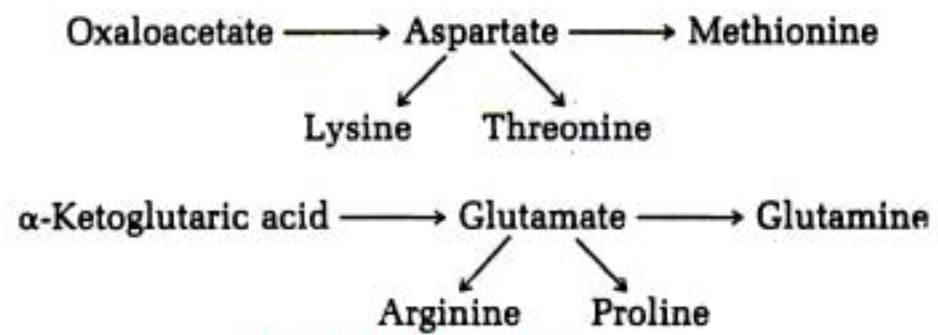




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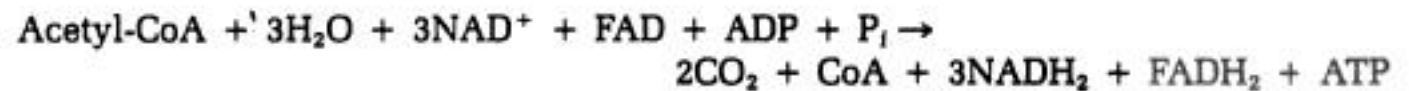


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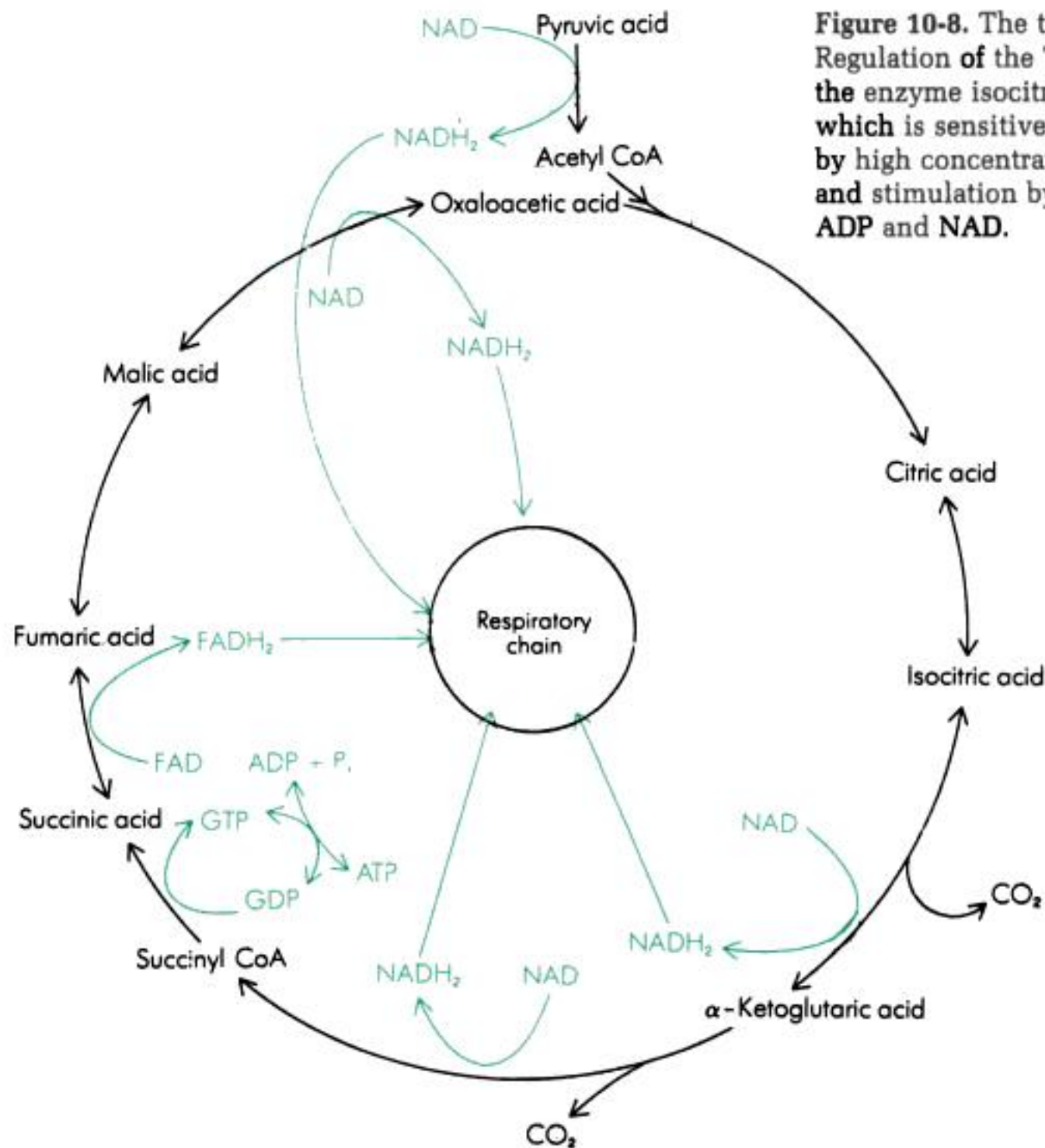


Thus the TCA cycle is an **amphibolic cycle**, which means that it functions not only in catabolic (breakdown) but also in anabolic (synthesis) reactions. The cycle is shown in Fig. 10-8.

The overall reaction of the TCA cycle can be summarized as follows:

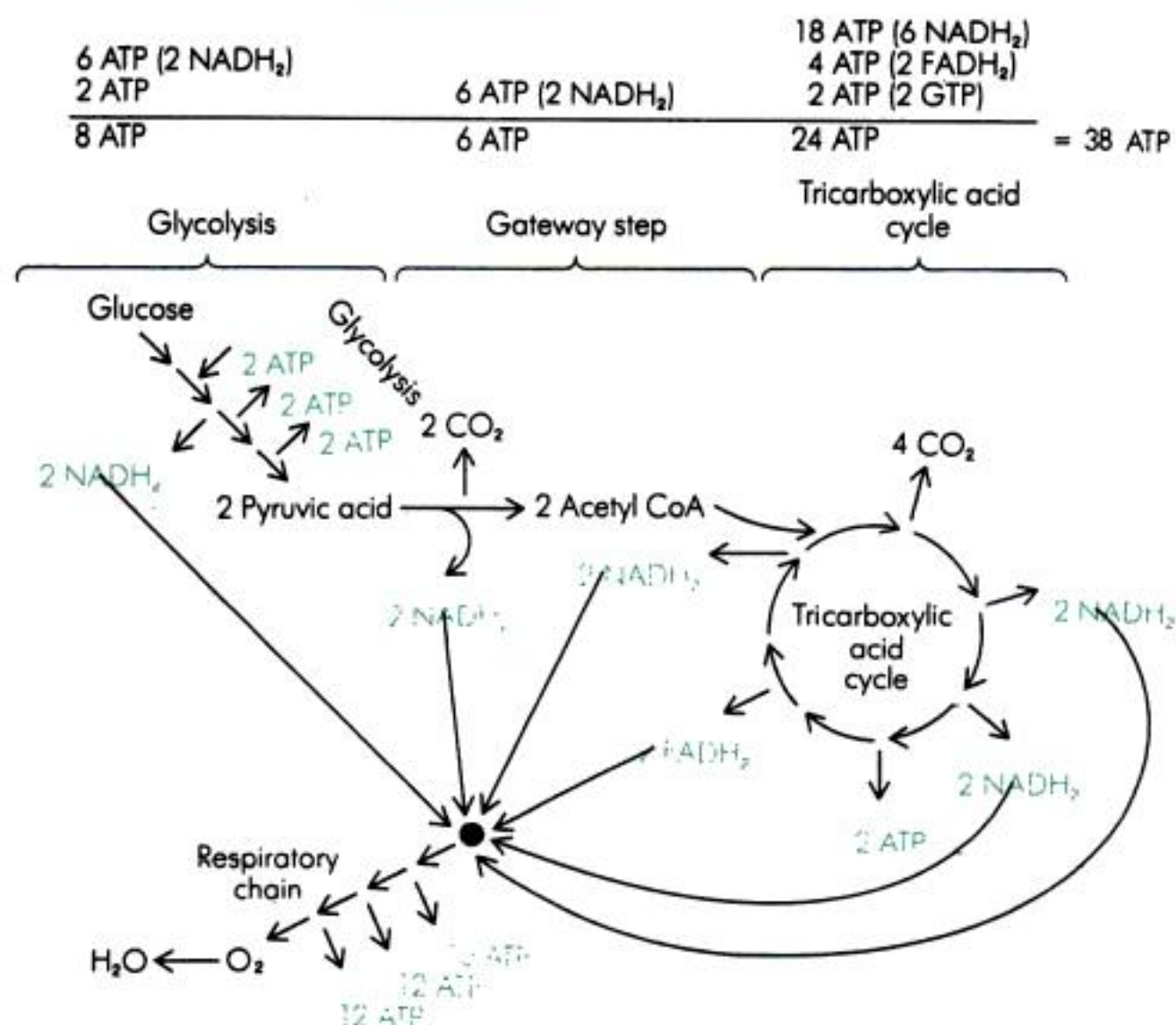


Since the breakdown of glucose by glycolysis yields two acetyl-CoA molecules



**Figure 10-8.** The tricarboxylic acid cycle. Regulation of the TCA cycle focuses on the enzyme isocitrate dehydrogenase, which is sensitive to feedback inhibition by high concentrations of ATP and NADH<sub>2</sub> and stimulation by high concentrations of ADP and NAD.





**Figure 10-9.** ATP yield per glucose molecule broken down in aerobic respiration.

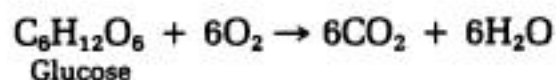
which can enter this cycle, the overall equation for the cycle, per glucose molecule broken down, is twice the above.

### Energy Yield in Aerobic Respiration

We may now look at the energy yield from the aerobic breakdown of one molecule of glucose when the electrons stored in the reduced coenzyme molecules are fed into the electron-transport chain. As shown previously, the electrons are transferred stepwise from the coenzyme carriers to molecular oxygen, and this transfer is coupled to the generation of ATP by oxidative phosphorylation.

For each glucose molecule broken down, there are 12 reduced coenzymes to be oxidized: 2 FADH<sub>2</sub> (1 from each turn of the TCA cycle) and 10 NADH<sub>2</sub> (2 from glycolysis; 2 from the gateway step between glycolysis and the TCA cycle, i.e., pyruvic acid to acetyl-CoA; and 6 from two turns of the TCA cycle). Since 3 ATP are produced from each NADH<sub>2</sub> and 2 ATP from each FADH<sub>2</sub>, there are 34 ATP generated from the reduced coenzymes via oxidative phosphorylation through the respiratory chain. But the total yield of ATP from the aerobic respiration of 1 glucose molecule is 38: 34 from the oxidation of reduced coenzymes, 2 from glycolysis, and 2 from the side reaction of the TCA cycle, that is, from 2 GTP. The total ATP yield per glucose molecule from aerobic respiration is summarized in Fig. 10-9.

The complete oxidation of glucose via glycolysis, the TCA cycle, and the respiratory chain is summarized in this overall reaction:

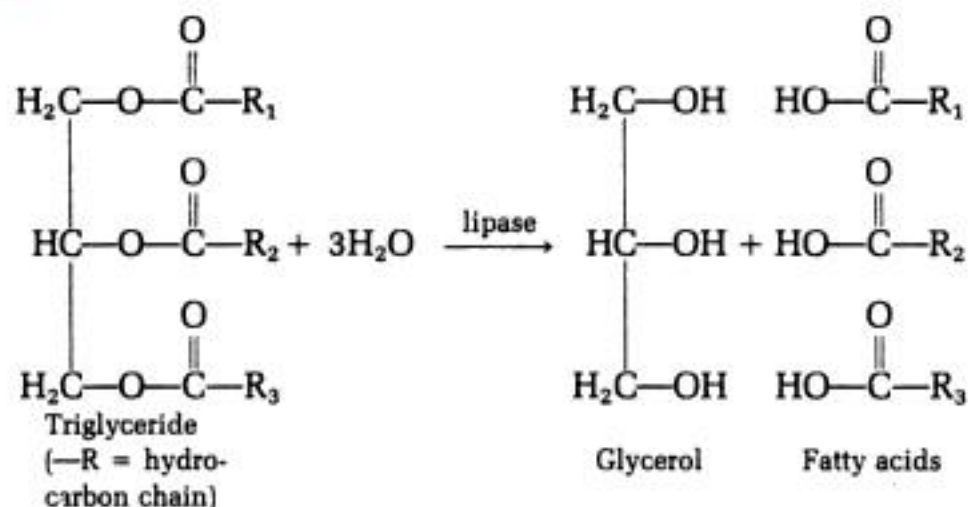




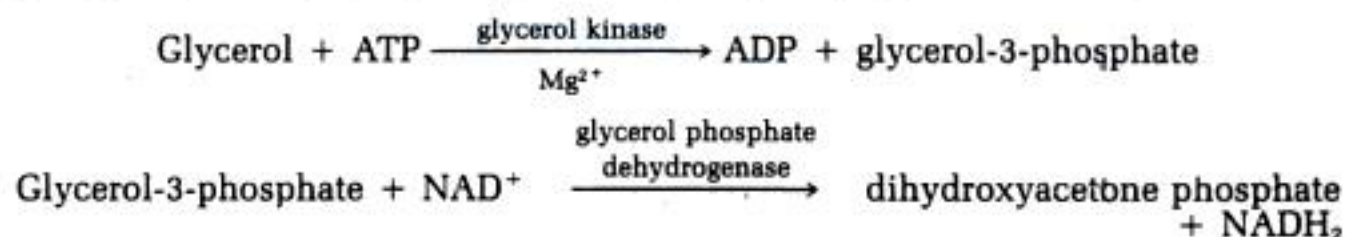
## Catabolism of Lipids

Glucose is the single most important source of energy for most cells. However, for many microorganisms, other substances, such as lipids and proteins, may be used as alternate sources of energy. There is a general rule that governs their utilization: they are converted as quickly and efficiently as possible into intermediates of the glycolytic and TCA pathways so that a minimum number of additional enzymes is required to effect complete breakdown. This rule highlights the fact that the glycolytic pathway and the TCA cycle serve as a common center around which other catabolic pathways are built.

The breakdown of lipids or fats begins with the cleavage of triglycerides by the addition of water to form glycerol and fatty acids by means of enzymes called **lipases**:



Glycerol as a component of fats can be converted into an intermediate of the glycolytic pathway (dihydroxyacetone phosphate) by the following reactions:



The dihydroxyacetone phosphate formed would be broken down by the mechanisms shown in Fig. 10-3. Fatty acids are oxidized by the successive removal of 2-carbon fragments in the form of acetyl-CoA, a process known as  **$\beta$ -oxidation**. The acetyl-CoA formed can then enter the TCA cycle, and the hydrogen atoms and their electrons enter the respiratory transport chain, leading to oxidative phosphorylation.

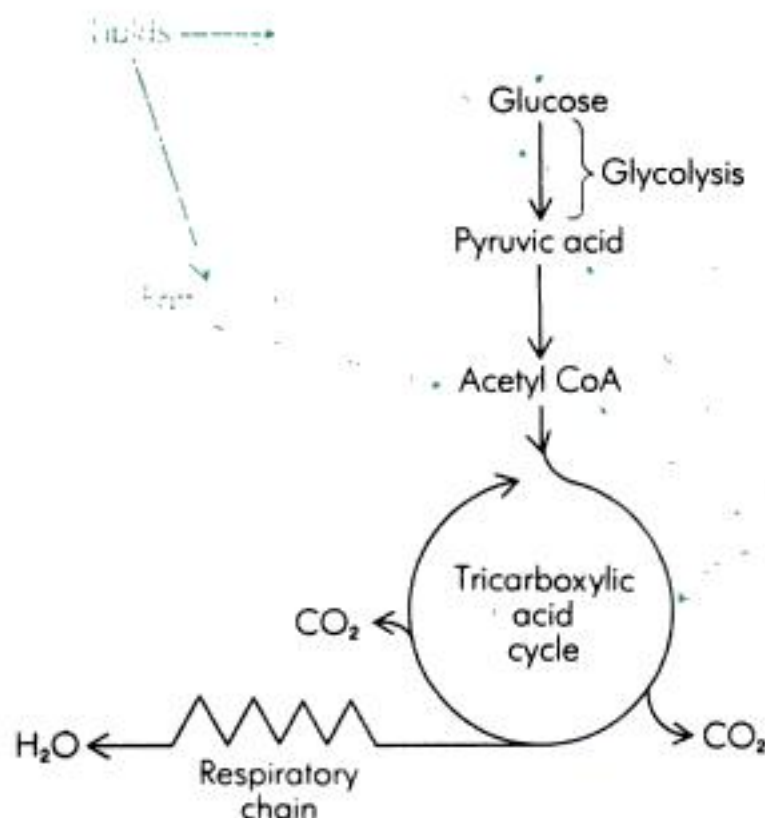
There is more energy yield per gram of fat than per gram of carbohydrate. However, relatively few microbial species are effective in breaking down lipids of either simple or complex types, partly because of the limited solubility of lipids.

## Catabolism of Proteins

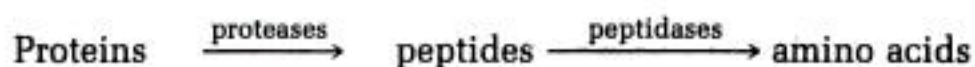
Many heterotrophic microorganisms can degrade exogenous proteins, using the products as carbon and nitrogen energy sources. Since protein molecules are too large to pass into the cell, bacteria secrete exoenzymes called proteases that hydrolyze exogenous proteins to peptides, which are then transported into the cell cytoplasm.



**Figure 10-10.** Metabolism of carbohydrates, lipids, and amino acids. As can be seen from the diagram, acetyl-CoA is a common intermediate of carbohydrate and lipid metabolism, and the TCA cycle is the common pathway for oxidation of carbohydrates, lipids, and amino acids.



Bacteria produce peptidases that break down peptides to the individual amino acids, which are then broken down according to the specific amino acid and the species or strain of bacteria breaking it down. This process may be shown as follows:



Where amino acids are broken down, the carbon skeletons of the amino acids undergo oxidation to compounds that may enter the TCA cycle for further oxidation. Entry into the TCA cycle can be via acetyl-CoA,  $\alpha$ -ketoglutaric acid, succinic acid, fumaric acid, or oxaloacetic acid.

An overall view of the dissimilation of carbohydrates, lipids, and proteins is shown in Fig. 10-10.

### Respiration without Oxygen in Some Bacteria

Some bacteria which are ordinarily aerobic can grow anaerobically if nitrate is present. For example, *Aquaspirillum itersonii*, an aquatic bacterium, is dependent on oxygen unless potassium nitrate is added to the medium. In such cases nitrate essentially substitutes for oxygen as the final electron acceptor in the respiratory chain. This process is termed **anaerobic respiration**. The pathways for the dissimilation of the carbon and energy sources are identical with those in aerobic respiration, and electron transport occurs via a respiratory chain similar to that in aerobic cells. Oxygen is replaced as the terminal electron acceptor by nitrate. However, in some strict anaerobes, other compounds, such as carbon dioxide, or ions, such as sulfate ion, can be the terminal electron acceptors.

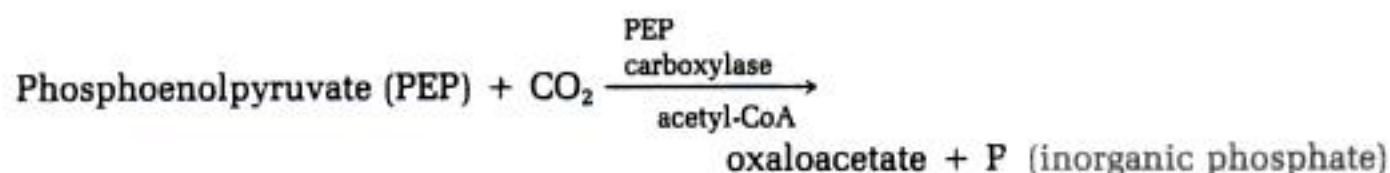
### Heterotrophic CO<sub>2</sub> Fixation

This phenomenon (unrelated to autotrophic CO<sub>2</sub> fixation) is important because it provides a mechanism for synthesis of compounds of the TCA cycle from the

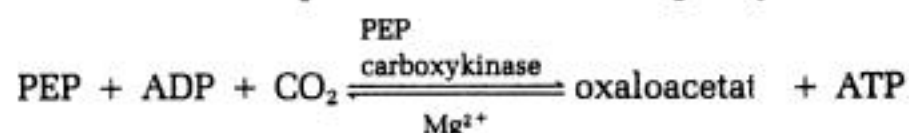


products of carbohydrate metabolism. Two types of  $\text{CO}_2$ -fixing reactions occur in heterotrophic bacteria.

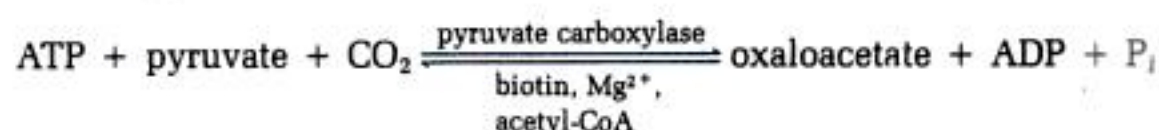
1 This first type of reaction is essentially irreversible and occurs in many bacteria:



A variation of this reaction requires a nucleoside diphosphate:



2 The second type requires the vitamin biotin for activity:

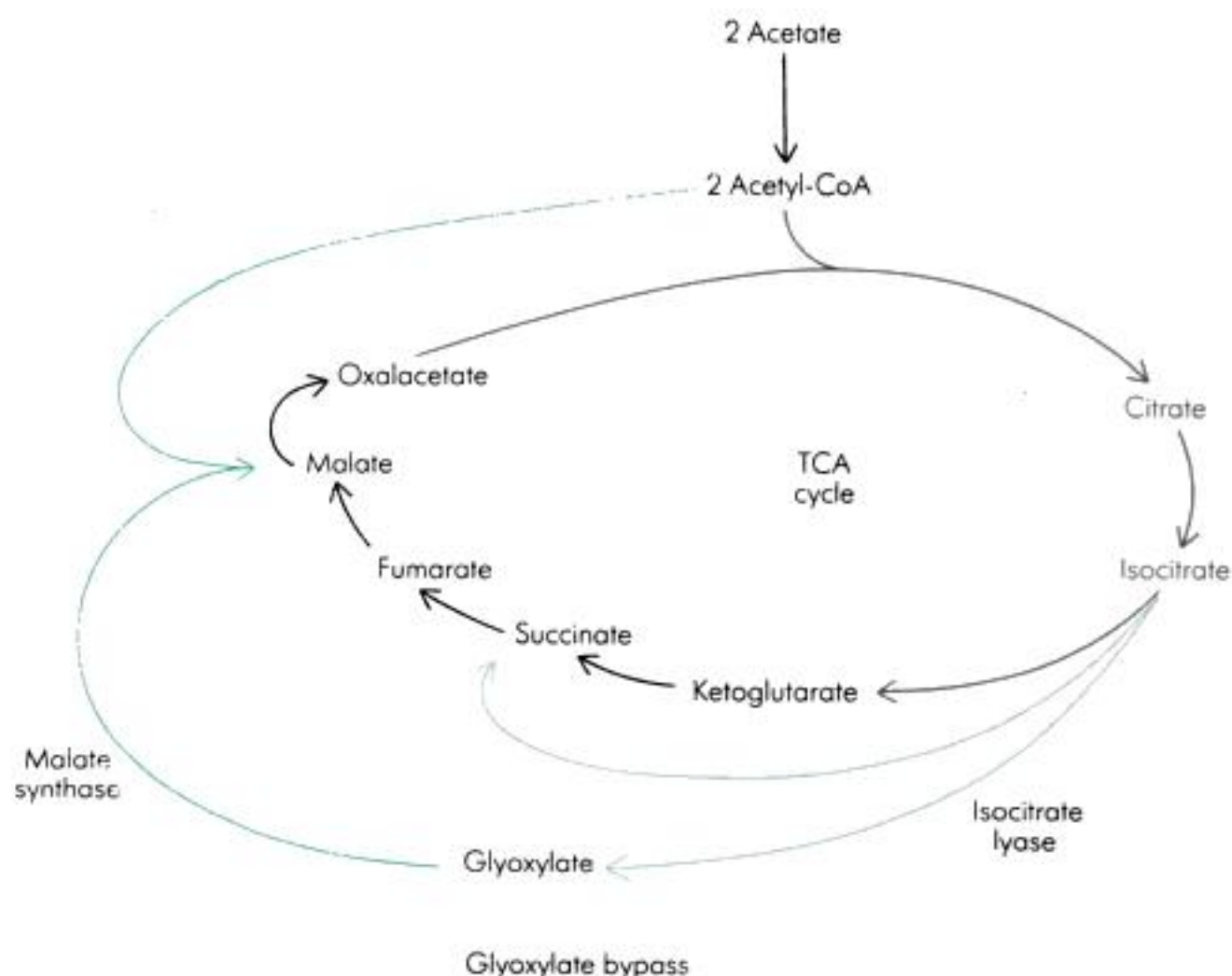


## The Glyoxylate Cycle

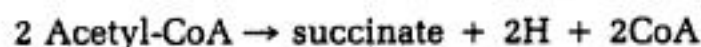
The glyoxylate cycle is used by some microorganisms when acetate is the sole carbon source or during oxidation of primary substrates (such as higher fatty acids) that are cleaved to acetyl-CoA without the intermediate formation of pyruvic acid. This pathway does not occur in higher organisms because they are never forced to feed on 2-carbon molecules alone.

The specific enzymes of the glyoxylate cycle are isocitrate lyase and malate synthase. Figure 10-11 shows how these two enzymes fit together with other

**Figure 10-11.** The glyoxylic acid cycle or bypass. Its reactions permit the replenishment of the pool of intermediates of the TCA cycle. The specific enzymes are isocitrate lyase and malate synthase.



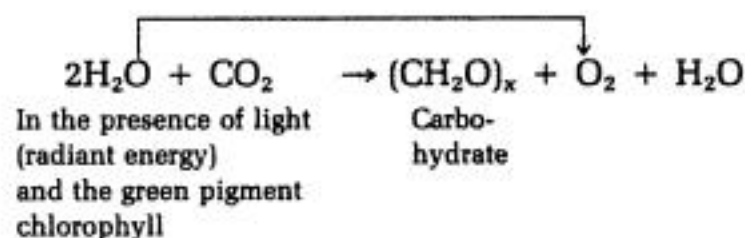
reactions of the tricarboxylic acid cycle to provide a bypass around some of the TCA-cycle reactions. The overall reaction of the glyoxylate cycle is



As seen in Fig. 10-11, acetyl-CoA enters the cycle at two places. It condenses with oxalacetate to give citrate, which is the entry point for the TCA cycle, and the further reaction leads to the formation of isocitrate. Isocitrate lyase is a splitting enzyme that produces succinate and glyoxylate. The second acetyl-CoA molecule condenses with glyoxylate to give malate by the action of malate synthase. Enzymes which carry out replenishment reactions such as this are known as *anaplerotic* enzymes; their function is to maintain the pool of essential intermediates for biosynthesis.

## ENERGY PRODUCTION BY PHOTOSYNTHESIS

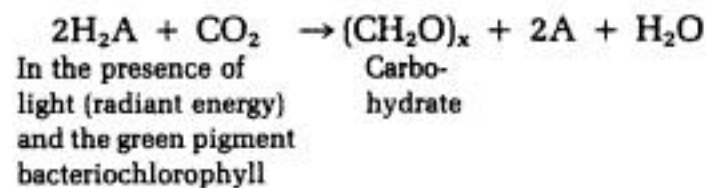
Plants, algae, and cyanobacteria are photoautotrophs. They use light as their source of energy and carbon dioxide as their sole source of carbon. In order for carbon dioxide to be useful for metabolism, it must first be reduced to carbohydrate. This process, by which light is used to convert carbon dioxide to carbohydrate, is called *photosynthesis*. The overall reaction can be written as



Here  $(\text{CH}_2\text{O})_x$  is a formula representing any carbohydrate.

Photosynthesis has two important requirements: (1) a large amount of energy in the form of ATP, and (2) a large quantity of a chemical reductant, in this case water.

Several groups of bacteria—the photoautotrophic green and purple bacteria—are also characterized by their ability to perform photosynthesis. But unlike plants, algae, and cyanobacteria, they do not use water as their chemical reductant, nor do they produce oxygen as one of their end products of photosynthesis. The general equation for bacterial photosynthesis is:



Here  $\text{H}_2\text{A}$  represents the chemical reductant, such as the inorganic compounds  $\text{H}_2$ ,  $\text{H}_2\text{S}$ , or  $\text{H}_2\text{S}_2\text{O}_3$ , or the organic compounds lactate or succinate. If  $\text{H}_2\text{A}$  in this equation stood for  $\text{H}_2\text{S}$ , then A would stand for S.

Both of the preceding equations represent the overall results of photosynthesis. A great deal has been learned about the specific chemical reactions involved in bacterial and plant photosynthesis. What follows is a look at the light-dependent energy-yielding processes involving bacteriochlorophyll in bacteria and chlorophyll in plants, algae, and cyanobacteria. What is presented is



### Cyclic and Nongcyclic Photophosphorylation

in accord with the latest results of many investigators but may require modification as further evidence is accumulated.

Anoxygenic photosynthetic bacteria possess chlorophylls, called bacteriochlorophylls, that differ from the chlorophylls of plants in structure and in light-absorbing properties. Bacteriochlorophylls absorb light in the infrared region (725 to 1,035 nm). They are not contained in chloroplasts but are found in extensive membrane systems throughout the bacterial cell.

When a molecule of bacteriochlorophyll absorbs a quantum of light, the energy of the light raises the molecule to an excited state. In this excited state an electron is given off by bacteriochlorophyll. Bacteriochlorophyll thus becomes positively charged. It then serves as an electron trap or strong oxidizing agent.

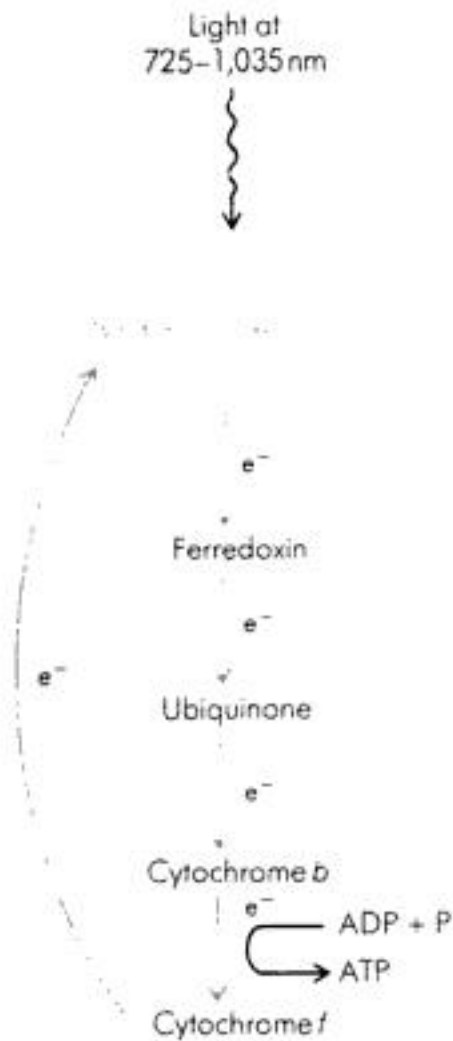
The electron, carrying some of the energy absorbed from light, is transferred to an iron-containing heme protein known as ferredoxin. From there it is passed successively to ubiquinone, to cytochrome *b*, and to cytochrome *f*, and finally back to the positively charged bacteriochlorophyll. Essentially, the electron has gone around in a cycle, beginning with, and returning to, bacteriochlorophyll. This relatively simple process is illustrated in Fig. 10-12.

The energy released in the step between cytochrome *b* and cytochrome *f* is used for photophosphorylation—the generation of ATP from ADP and inorganic phosphate.

Note that no  $\text{NADP}^+$  has been reduced in these reactions. The reduction of  $\text{NADP}^+$  in photosynthetic bacteria is accomplished not by photosynthesis but by using reducing power from constituents of the environment, such as  $\text{H}_2\text{S}$  and other inorganic and organic compounds. Such reduced compounds usually abound in the anaerobic environment of photosynthetic bacteria.

It may be added that light of higher energy than that absorbed by bacteriochlorophylls can contribute to bacterial photosynthesis since there are carotenoids and other accessory pigments in the bacterial cells which absorb light at shorter wavelengths and transfer the energy to the bacteriochlorophylls.

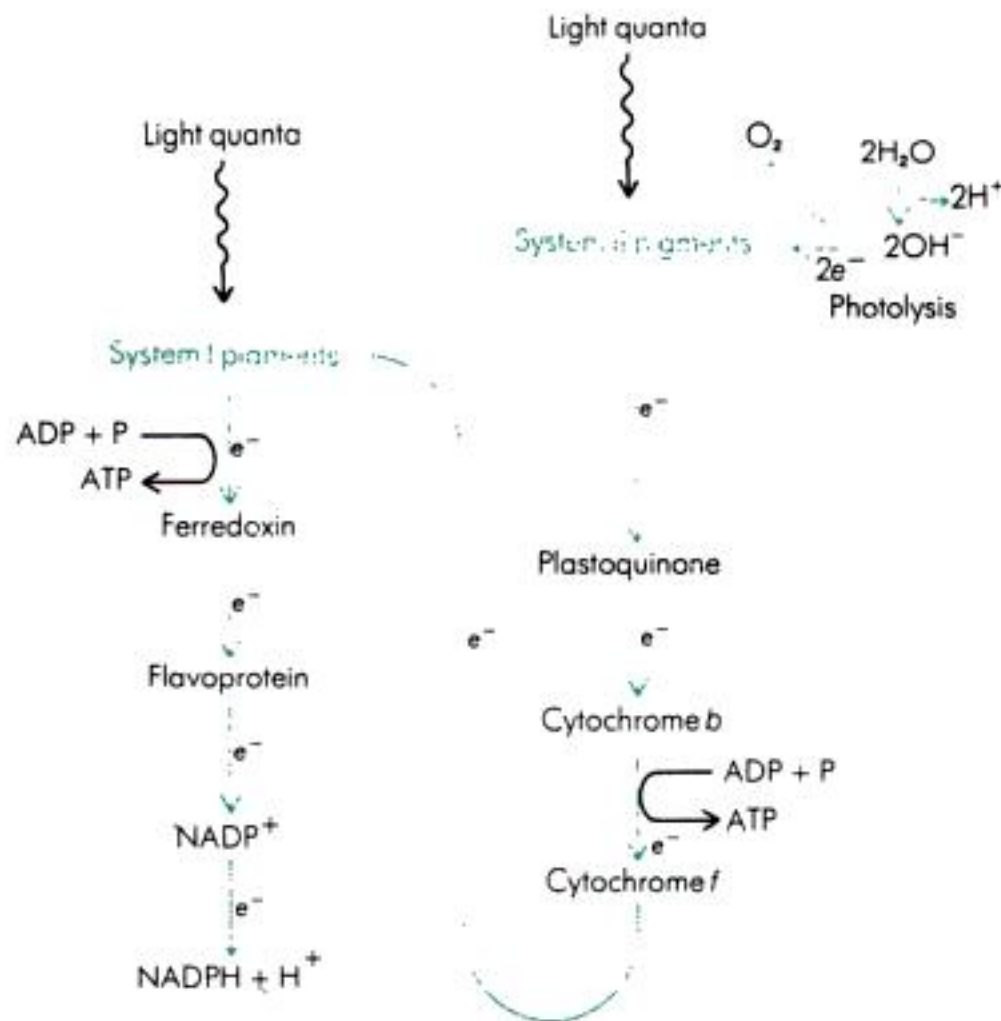
In plants, algae, and cyanobacteria (oxygenic photosynthetic bacteria), nongcyclic photophosphorylation occurs in photosynthesis. In this process, when a molecule in pigment system II (one of two systems of light reactions) absorbs light, this energy raises the molecule to an excited state and the molecule releases an electron. This electron is transferred to plastoquinone, to cytochrome *b*, to cytochrome *f*, and finally to pigment system I. Photophosphorylation occurs with generation of ATP from ADP and inorganic phosphate in the step between cytochrome *b* and cytochrome *f*. When pigment system I absorbs light, it releases an electron. This electron is transferred from ferredoxin, to flavoprotein, to  $\text{NADP}^+$ . Photophosphorylation occurs again between the release of the electron from pigment system I to ferredoxin. Also note that  $\text{NADP}^+$  is reduced in this part of the process (see Fig. 10-13). This process differs from cyclic photophosphorylation because the electron lost by pigment system II is not cycled back to it. Instead, electrons are replaced in pigment system II by the light-generated breakdown of water, called *photolysis*. There is some evidence that this scheme of nongcyclic photophosphorylation, shown in Fig. 10-13, may have to be modified. It appears that system II pigments alone can carry out the entire process



**Figure 10-12.** Cyclic photophosphorylation as it occurs in anoxygenic photosynthetic bacteria. The electron returns, at a lower energy state, to the bacteriochlorophyll, which had become positively charged after the initial ejection of the electron. No  $\text{NADP}$  is reduced and no external donor is necessary for this process.



**Figure 10-13.** Noncyclic photophosphorylation as it occurs in green plants, algae, and cyanobacteria. In this process, electrons raised to a high energy state ultimately reduce  $\text{NADP}^+$  and are not recycled to the light-pigment systems. The protons necessary for reduction come from the dissociation of water, which results in evolution of oxygen. Electrons are restored to the pigments of system II from the  $\text{OH}^-$  ion of  $\text{H}_2\text{O}$ . The  $\text{OH}^-$  ion is split to  $e^-$ ,  $\text{H}^+$ , and  $\frac{1}{2} \text{O}_2$  by photolysis.

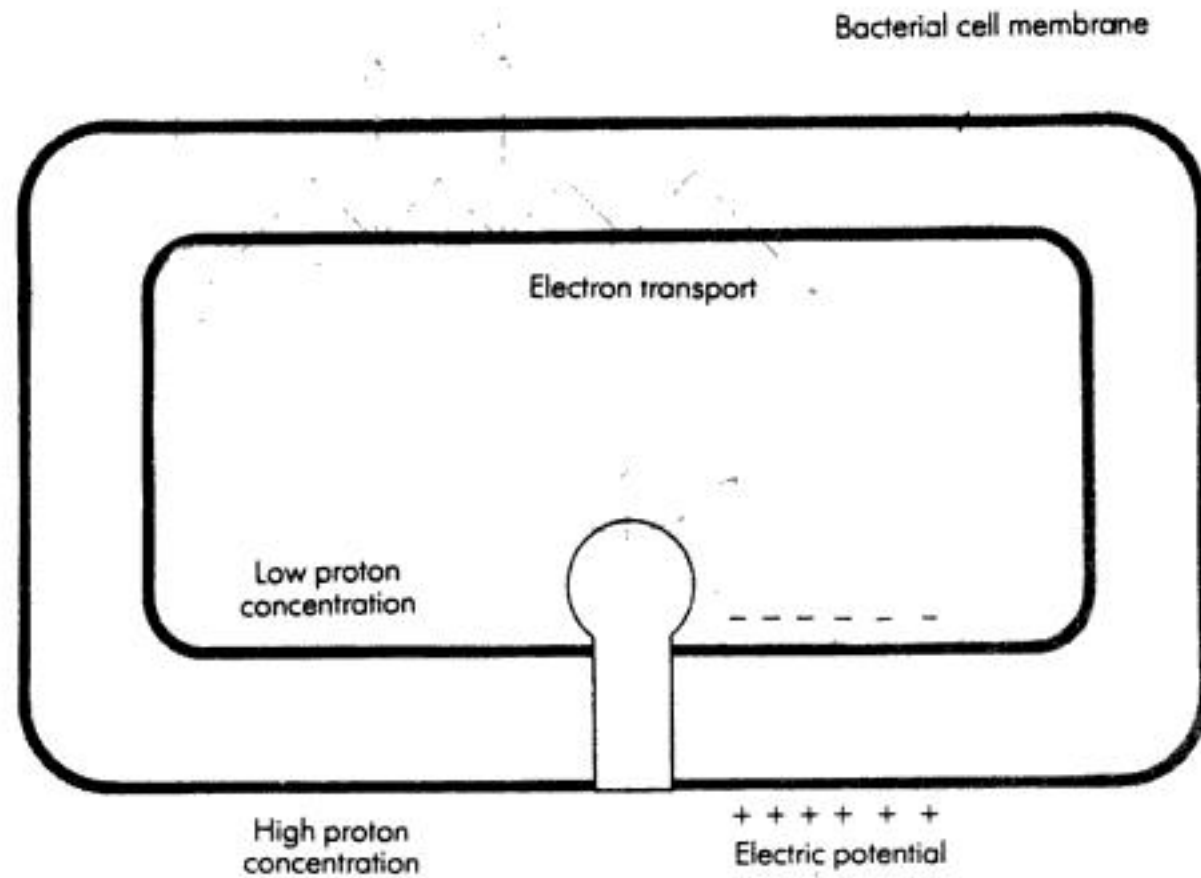


of noncyclic photophosphorylation. Thus the noncyclic reduction of ferredoxin need not involve system I pigments. Further, the most important role of plastoquinone is in the transport of protons originating from water. This modified process has been termed **oxygenic photophosphorylation**. Further evidence is needed for its confirmation.

## THE MECHANISM OF ATP SYNTHESIS

The chemical reactions that lead to the synthesis of ATP are now well understood. But how the transfer of electrons through the respiratory transport chain is coupled to the synthesis of ATP is not very clear. Several alternate hypotheses have been proposed to explain how energy released during electron transport is conserved in the form of ATP. The prevailing theory is the **chemiosmotic hypothesis** advanced in 1961 by Peter Mitchell, a British biochemist. Mitchell was awarded the Nobel prize for his work in this field in 1978. According to this theory, the flow of electrons through the system of carrier molecules releases energy which drives positively charged hydrogen ions ( $\text{H}^+$ ), or protons, across the membranes of chloroplasts, mitochondria, and bacterial cells (Fig. 10-14). This movement of hydrogen ions results in the acidification of the surrounding medium and the generation of a **pH gradient** (a difference in pH) across the organelle or cell membrane. In addition, such hydrogen-ion movements lead to the formation of an **electric potential gradient** (a difference in charge) across the membrane (since an electric charge is carried by the proton). In this way, energy released during the transfer of electrons through the respiratory chain is conserved as a "protonmotive force"; the electric potential gradients are produced by pumping hydrogen ions across the membrane.

**Figure 10-14.** Mechanism of ATP synthesis. Flow of electrons through the respiratory chain drives hydrogen ions across the membrane. This results in a high hydrogen-ion concentration outside the cell and a low concentration inside the cell. This produces a pH and electrochemical gradient. ATP synthesis at the site of the ATPase complex (a knobbed structure on the membrane) is driven by the release of energy when hydrogen reenters the bacterial cell.



Following this first energy-conservation step, when the hydrogen ions reenter the organelle or cell, they are transported by the membrane-bound enzyme adenosine triphosphatase. The energy released on reentry drives the synthesis of ATP, the second energy-conservation step. This process is shown in Fig. 10-14.

## QUESTIONS

- What is meant by the following?
  - Free-energy change
  - Exergonic reaction
  - Endergonic reaction
  - $\Delta G$ ,  $\Delta G^\circ$ ,  $\Delta G'^\circ$
- Explain the relationship between  $\Delta G^\circ$  and the equilibrium constant, and the relationship between  $\Delta G^\circ$  and an oxidation-reduction potential difference.
- What role does ATP play in energy exchanges in cells?
- In what way is the coupling of exergonic reactions with endergonic reactions important in living organisms?
- Define the meaning of a high-energy-transfer compound. Name those that occur in the glycolytic pathway.
- Explain what is meant by an oxidation-reduction system.
- What is oxidative phosphorylation? Where does it occur in the respiratory chain?
- Briefly explain how glycolysis fits into the metabolism of glucose in aerobic cells.
- Compare the disposition of electrons (or hydrogen atoms) obtained from the oxidation of glyceraldehyde-3-phosphate in aerobic and anaerobic cells.
- Identify the three reactions in the glycolytic pathway that are not freely reversible by the same specific enzymes.



- 11 Account for the ATP yield per glucose molecule in glycolysis.
- 12 Describe the various ways in which the pentose phosphate cycle is useful to a cell.
- 13 Is the Entner-Doudoroff pathway found in (a) both aerobes and anaerobes; (b) both eucaryotes and procaryotes?
- 14 Explain why fermentation is a less efficient process for obtaining energy than aerobic respiration.
- 15 Explain how fermentation products can be used for the identification of bacteria. Provide specific examples to support your answer.
- 16 Why is the TCA cycle called an amphibolic cycle?
- 17 What general rule governs the utilization of substances other than carbohydrates for the production of energy?
- 18 Name the specific enzymes of the glyoxylate cycle and describe the reactions they catalyze.
- 19 What are the essential differences between photosynthesis by bacteria and by algae?
- 20 Explain why photophosphorylation in procaryotes is termed *cyclic*.
- 21 Describe the prevailing model for the mechanism of ATP synthesis.

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## Chapter 11

# Microbial Metabolism: Utilization of Energy and Biosynthesis

**OUTLINE** Utilization of Energy in Nonbiosynthetic Processes  
Bacterial Motility • Transport of Nutrients by Bacteria

### Utilization of Energy in Biosynthetic Processes

Synthesis of Small Molecules: The Amino Acids • Synthesis of Macromolecules: The Structure and Biosynthesis of a Cell-Wall Peptidoglycan • Synthesis of Organic Cell Material in Chemoautotrophic Bacteria

### The Biosynthesis of Deoxyribonucleic Acid

The Structure of Deoxyribonucleic Acid • The Structure of Ribonucleic Acid • The Biosynthesis of Nucleotides in DNA Synthesis • Semiconservative Replication of DNA Strands • Replication of the DNA Molecule

### Transcription and Translation of Genetic Information

The Building Blocks of Proteins • Transcription • Translation

### The Process of Protein Synthesis

Assembly of the Protein Chain on the Ribosome

In the preceding chapter, we discussed some of the chemical mechanisms by which different organisms obtain energy. In this chapter, we provide the sequel by describing some of the ways in which energy, once obtained, is utilized by microorganisms. We shall look at some examples of how this is done.

As we have seen in Chap. 10, energy is stored in the form of high-energy-transfer compounds (of which ATP is the most important). But energy is also available in the form of the **protonmotive force** (or electrochemical proton gradient). In these terms, energy is used to drive the many endergonic reactions required for the life of the cell.

The principle of coupling exergonic reactions to endergonic reactions requires the utilization of high-energy transfer compounds like ATP. An electrochemical proton gradient may result in ATP synthesis (see Fig. 10-14), but it can also be used for other biological purposes without the synthesis of ATP. For example, it can be used to generate heat and the rotation of bacterial flagella.

The ATP formed by the energy-producing reactions of the bacterial cell is expended in various ways. Much of it is used in the biosynthesis of new cell components, including energy-storage inclusion granules such as glycogen and



poly- $\beta$ -hydroxybutyrate. Other metabolic processes which require phosphate-bound energy or the energy of the protonmotive force include maintenance of the physical and chemical integrity of the cell, transport of solutes across membranes, and activity of locomotor organelles.

Maintenance of the physical and chemical integrity of the cell is mainly through reactions that lead to biosynthesis of macromolecules, such as nucleic acids and proteins, that are continuously being broken down and need replacement. The extent of this degradation varies with the environmental conditions. Transport of solutes across membranes also requires energy, as does mechanical work such as motility by means of flagella. In general, the rate of utilization of energy in ATP determines the rate at which ATP is regenerated from ADP at the expense of energy from the environment.

## Bacterial Motility

Bacterial flagella filaments appear to have no machinery for interconverting chemical and mechanical energy. For example, **flagellin**, the flagellar protein molecule, has no enzymatic activity, i.e., no detectable ATPase activity (such as is present in cilia and flagella of eucaryotic microorganisms). Bacterial flagella thus differ markedly from the much larger and more complex cilia and flagella that propel eucaryotic cells such as protozoa.

It is therefore not surprising that ATP has been demonstrated not to be the immediate source of energy for flagella rotation. Instead the flagellar motor (that rotates the flagellum) is driven by the protonmotive force, i.e., the force derived from the electric potential and the hydrogen gradient across the cytoplasmic membrane.

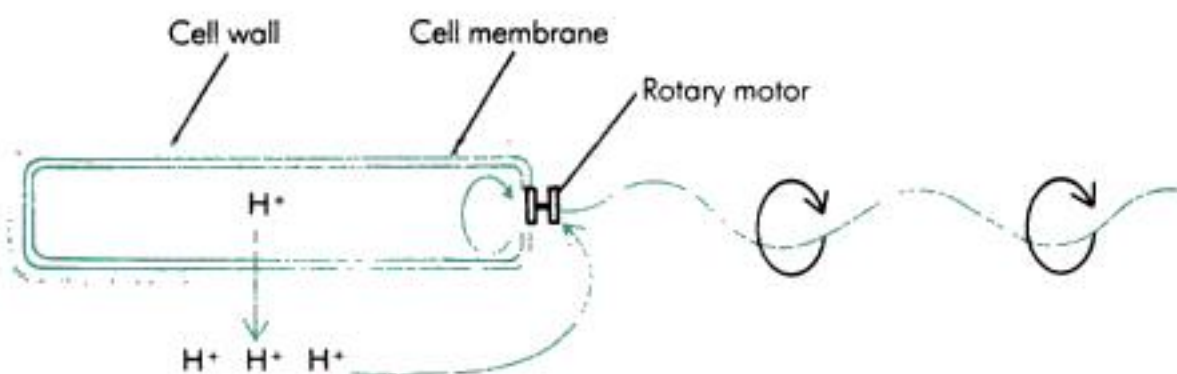
The rotary motor is believed to be the two elements in the basal body, the M ring and the S ring (see Chap. 5). The rod (which is connected to the filament by the hook) is fixed rigidly to the M ring, which rotates freely in the cytoplasmic membrane. The S ring is mounted rigidly on the cell wall. The inward flux of protons drives the flagellar motor (Fig. 11-1). Exactly what molecular events cause the conversion of protonmotive force into mechanical rotation are still unknown. However, it is clear that in the case of flagellar rotation, proton movements, and not ATP, constitute the energy currency.

## Transport of Nutrients by Bacteria

We shall now give an account of the various processes by which ions or molecules cross the cytoplasmic membrane. It is the cytoplasmic membrane that allows the passive passage of certain small molecules and actively concentrates others within the cell.

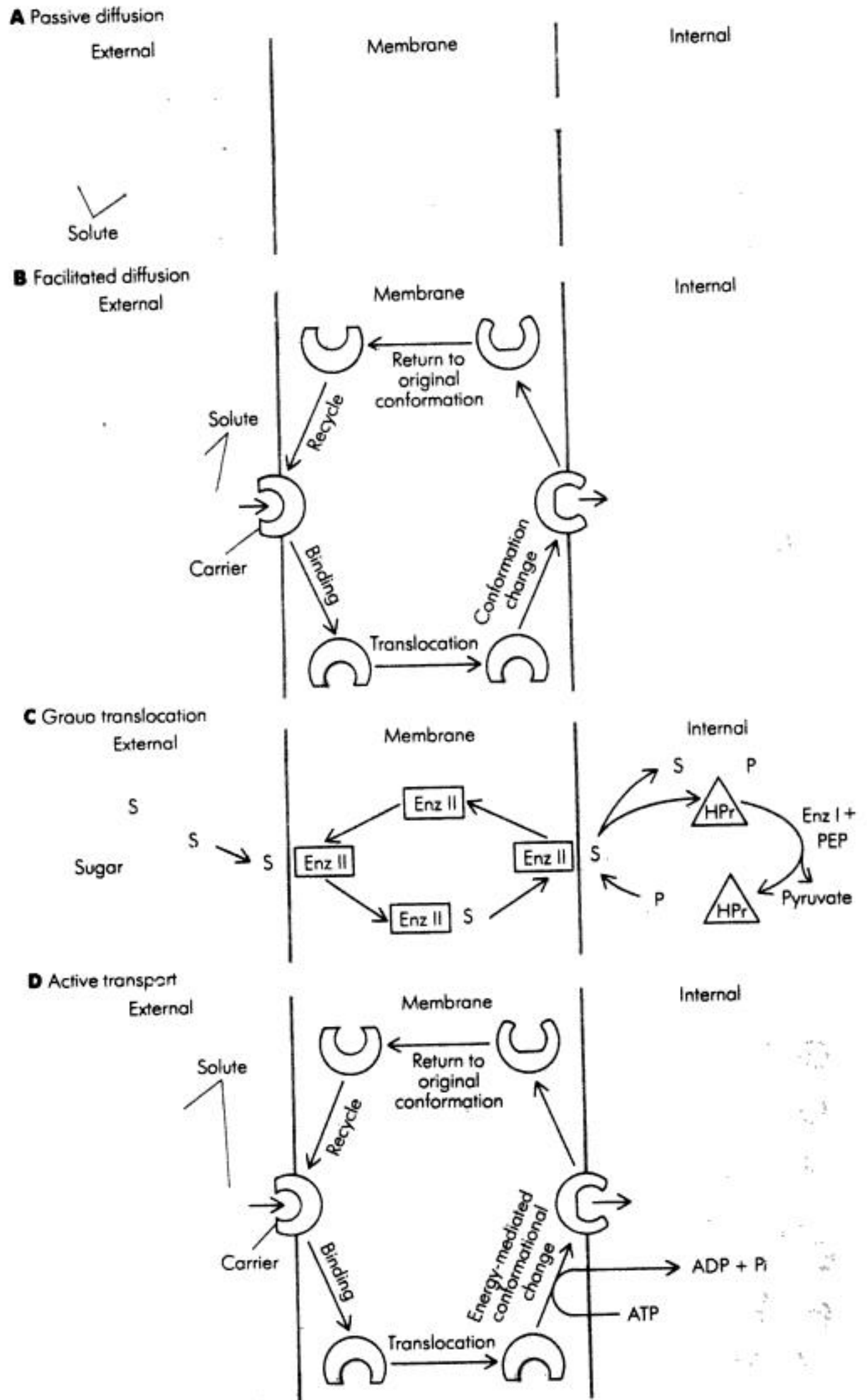
### Passive Diffusion

Except for water and some lipid-soluble molecules, few compounds can pass through the cytoplasmic membrane (a lipid-protein, semipermeable cell mem-



**Figure 11-1.** The flagellum of bacteria is driven by the protonmotive force. Protons flowing back into the cell through the basal body rings of each flagellum cause it to rotate; these rings constitute the rotary motor.

**Figure 11-2.** Mechanisms of nutrient transport into cells: (A) passive diffusion; (B) facilitated diffusion; (C) group translocation; (D) active transport.





brane) by simple or *passive diffusion*. In this process solute molecules cross the membrane as a result of a difference in concentration of the molecules across the membrane. The difference in concentration (higher outside the membrane than inside) governs the rate of inward flow of the solute molecule. With time, this concentration gradient diminishes until equilibrium is reached. In passive diffusion no substance in the membrane interacts specifically with the solute molecule as illustrated in Fig. 11-2A.

### Facilitated Diffusion

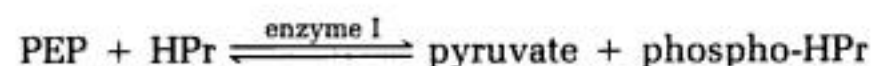
Another mechanism by which substances cross the semipermeable cell membrane is *facilitated diffusion*. This process is similar to passive diffusion in that the solute molecule also flows from a higher to a lower concentration. But it is different from passive diffusion because it involves a specific protein carrier molecule (called a *porter* or *permease*) located in the cytoplasmic membrane. The carrier molecule combines reversibly with the solute molecule, and the carrier-solute complex moves between the outer and inner surfaces of the membrane, releasing one solute molecule on the inner surface and returning to bind a new one on the outer surface. This process is shown in Fig. 11-2B. The entry of glycerol into bacterial cells is by facilitated diffusion. Although this mechanism of transport is common in eucaryotic cells (e.g., sugars enter them in this way), it is relatively rare in procaryotic cells. Neither of the above two mechanisms, passive diffusion or facilitated diffusion, require metabolic energy. Nor do they result in *concentration* or *accumulation* of solute against an electrochemical (with ions) or osmotic (with nonelectrolytes) gradient. Of greater interest to us in the context of this chapter are the two other mechanisms by which solutes cross membranes, both of which require metabolic energy and accumulate substrates against concentration gradients. Solute can be concentrated within the cell several thousand times greater than outside the cell. These two mechanisms are *group translocation* and *active transport*.

### Group Translocation

In group translocation the solute is altered chemically during transport. The best-studied group-translocation system is the *phosphotransferase system* (PTS). It is widely distributed in many bacterial genera and mediates the translocation of many sugars and sugar derivatives. These solutes enter the cell as sugar phosphates and are accumulated in the cell in this form.

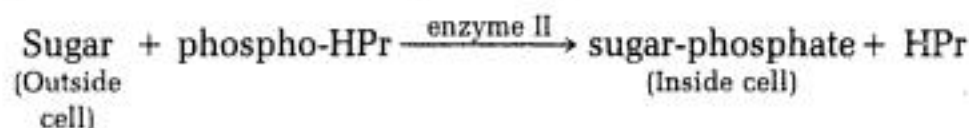
Phosphotransferase system (PTS) sugar uptake and phosphorylation require the participation of several soluble and membrane-bound enzymes. These proteins catalyze the transfer of the phosphoryl group of phosphoenolpyruvate to the sugar molecule. The products formed are therefore sugar phosphate and pyruvate; the overall reaction requires  $Mg^{2+}$ .

Specifically, a relatively heat-stable carrier protein (HPr) is activated first by transfer of a phosphate group from the high-energy compound phosphoenolpyruvate (PEP) inside the cell, as shown in Fig. 11-2C.



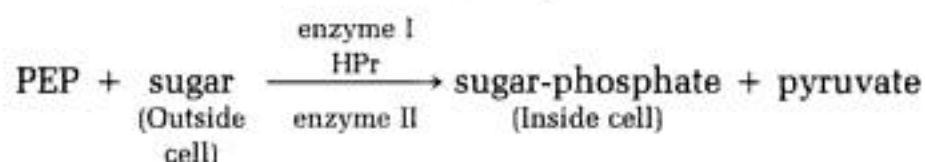
Enzyme I and HPr are soluble proteins and are nonspecific components of the process.

At the same time, the sugar combines with enzyme II at the outer membrane surface and is transported to the inner membrane surface (see Fig. 11-2C). Enzyme II is specific for a particular sugar and is an integral component of the cytoplasmic membrane. Here it combines with the phosphate group carried by the activated HPr. The sugar-phosphate is released by enzyme II and enters the cell. This is illustrated by the reaction equation shown below. Some investigators have reported a peripheral membrane enzyme III that mediates between enzyme II and phospho-HPr in translocating the sugar.



Enzyme I has been partially purified from several bacteria including *Escherichia coli* and *Salmonella typhimurium*. HPr has been purified to homogeneity from several bacteria. Mannitol enzyme II has been purified from *E. coli*.

The net chemical reaction of PTS sugar uptake is therefore:



Other known group-translocation processes include the uptake of adenine and butyrate at the exterior surface of the cell and their conversion at the interior membrane surface to adenosine monophosphate and butyryl-coenzyme A, respectively.

## Active Transport

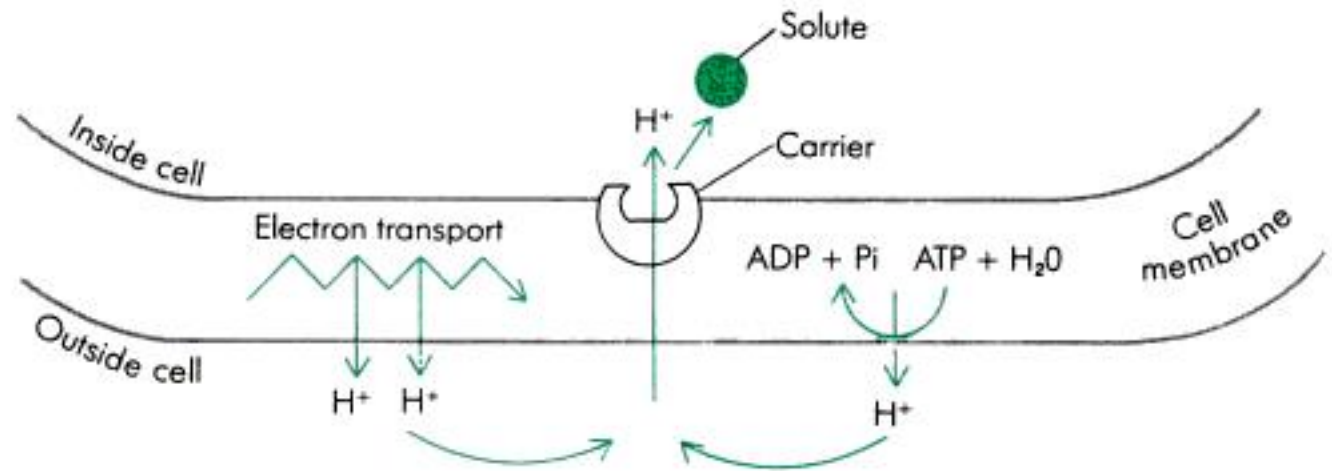
Almost all solutes, including sugars, amino acids, peptides, nucleosides, and ions, are taken up by cells through active transport. The three steps of active transport are:

- 1 Binding of a solute to a receptor site on a membrane-bound carrier protein.
- 2 Translocation of the solute-carrier complex across the membrane.
- 3 Coupling of translocation to an energy-yielding reaction to lower the affinity of the carrier protein for the solute at the inner membrane surface so that the carrier protein will release solute to the cell interior. This process is illustrated in Fig. 11-2D.

Several mechanisms have been proposed to explain the molecular basis of active transport of solutes in microorganisms. The accumulated evidence suggests that active transport may also be explained by Mitchell's chemiosmotic theory (see Chap. 10). In this case, energy released during the flow of electrons through the electron-transport chain or the splitting of a phosphate group from ATP drives protons out of the cell. This generates a difference in pH value and electric potential between the inside and the outside of the cell or across the membrane. This proton gradient gives rise to a protonmotive force which can be used to pump the solutes into the cell. When protons reenter the cell, the energy released on reentry drives the transport mechanism in the cell membrane, probably by inducing a conformational change in the carrier molecule so that its affinity for the solute is decreased and the solute is released into the cell interior. The link between active transport and metabolic energy generation is illustrated in Fig. 11-3.



**Figure 11-3.** How release of metabolic energy is coupled to and drives active transport.



Many active-transport systems of Gram-negative bacteria are associated with **binding proteins** in the periplasmic space. These binding proteins have very high affinities for specific nutrients, including amino acids, sugars, and inorganic ions. Over one hundred different binding proteins have been isolated and characterized. They are essential for active transport of their specific substrates. However, they are not porters since they are located in the periplasmic space rather than in the cell membrane itself. But binding proteins function in conjunction with porters in the active transport of specific nutrients.

## UTILIZATION OF ENERGY IN BIOSYNTHETIC PROCESSES

We have seen how energy is utilized for motility and transport of nutrients into bacterial cells. These are **nonbiosynthetic** processes. **Biosynthetic** processes in the cell also require energy; energy from ATP is used to convert one chemical substance into another and to synthesize complex substances from simpler ones.

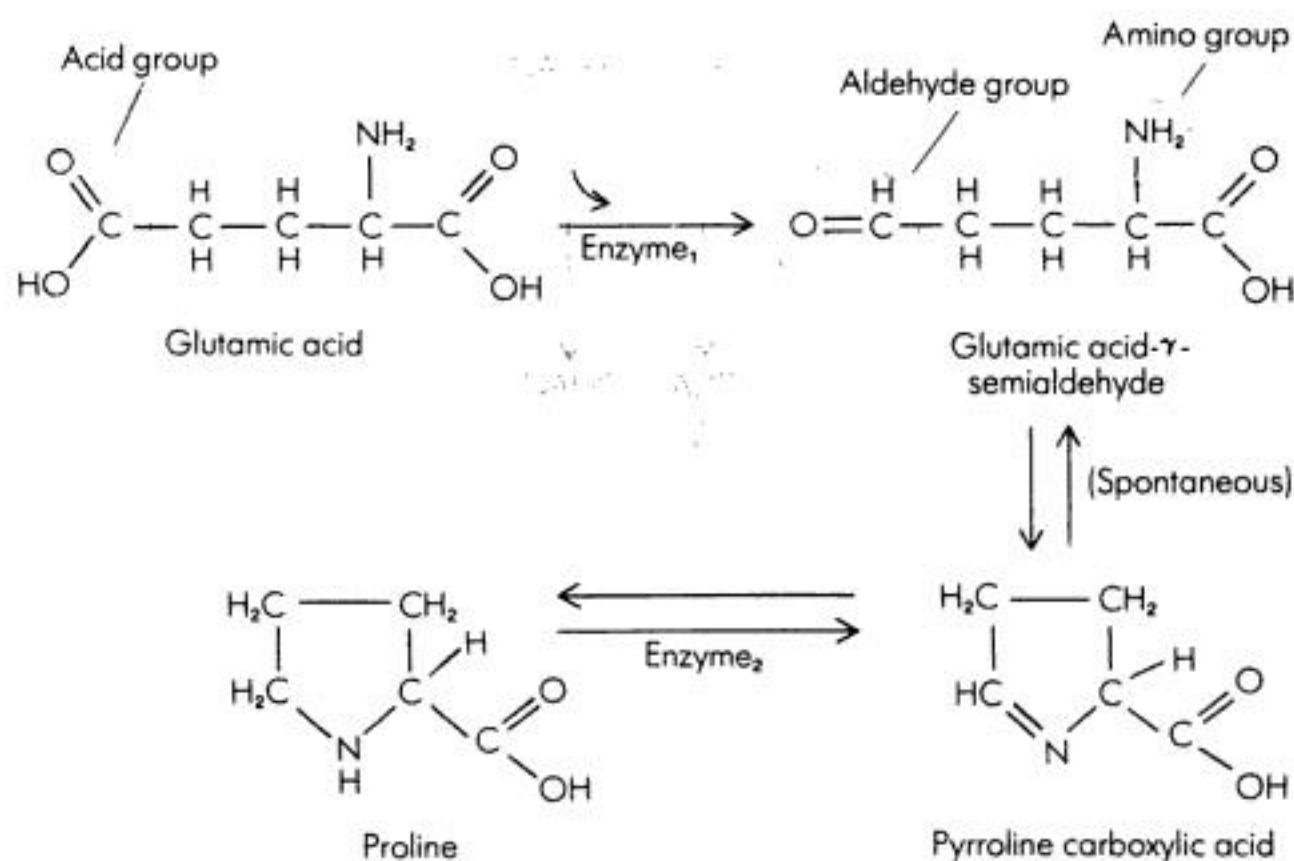
### Synthesis of Small Molecules: The Amino Acids

Amino acids, of which there are about 20 (Table 11-1), are the building blocks of proteins. The sequence and manner in which they are linked (i.e., their three-dimensional structure) determine the type of protein they form.

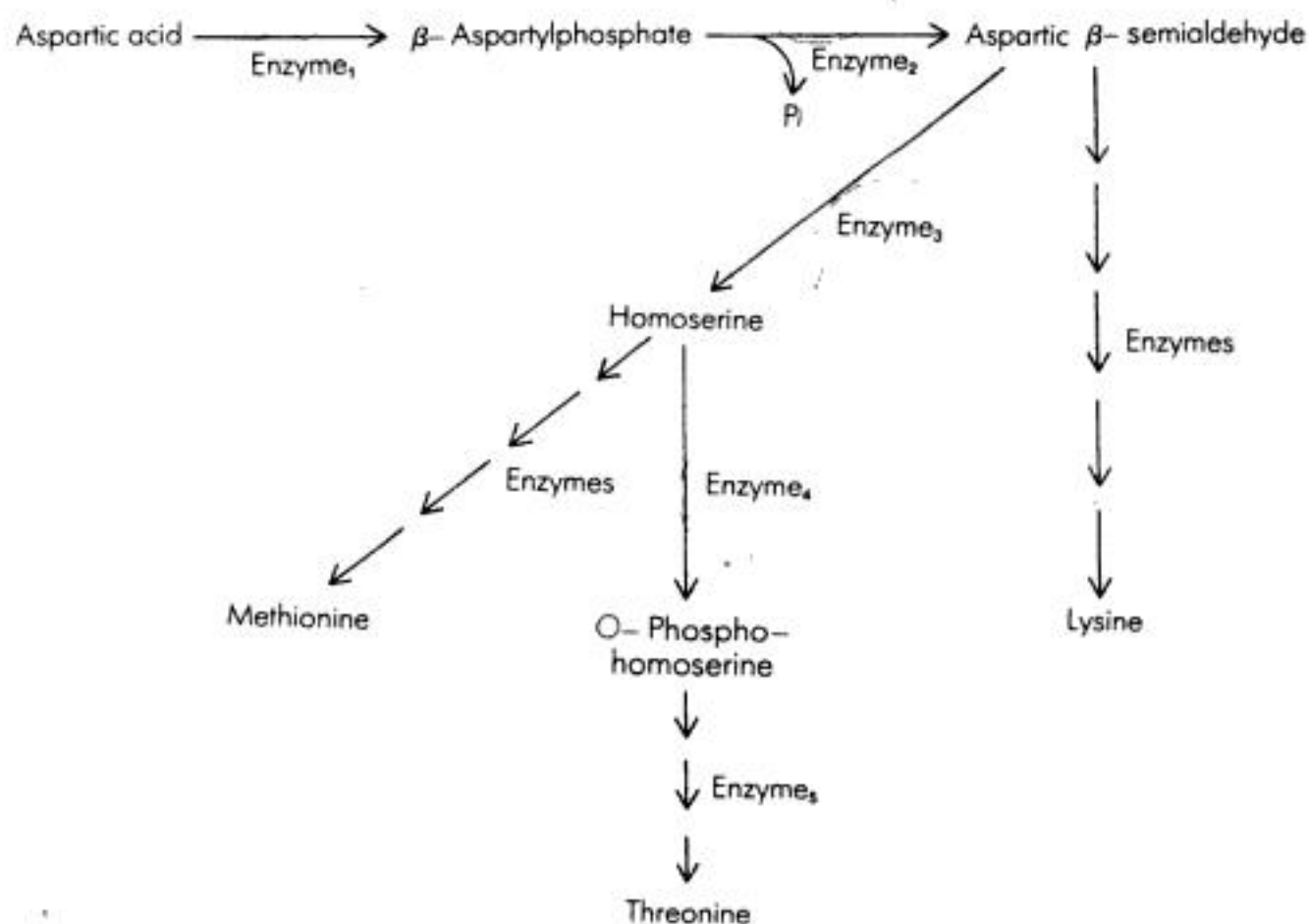
A microorganism growing in a medium may have all 20 of the amino acids present in the medium; that is, they are available for the microbe, preformed in the medium. If they are not available freely in the medium, the microorganism may have to liberate amino acids from proteins by the action of intracellular or extracellular proteolytic enzymes. In this way, the amino acids become available for use as nutritional building blocks. Sometimes, only a few amino acids are present in a medium, in which case the microbe has to convert other amino acids from the available ones into those that are missing. In yet other instances, the medium contains only inorganic sources of nitrogen, such as ammonium salts. The microorganism then has to synthesize all the required amino acids from these sources of available nitrogen, provided, of course, that it has this

**Table 11-1.** Amino Acid Building Blocks of Protein, with Standard Abbreviations

Alanine (Ala)	Glycine (Gly)	Proline (Pro)
Arginine (Arg)	Histidine (His)	Serine (Ser)
Asparagine (Asp-NH <sub>2</sub> , Asn)	Isoleucine (Ile)	Threonine (Thr)
Aspartic acid (Asp)	Leucine (Leu)	Tryptophan (Trp)
Cysteine (Cys)	Lysine (Lys)	Tyrosine (Tyr)
Glutamic acid (Glu)	Methionine (Met)	Valine (Val)
Glutamine (Glu-NH <sub>2</sub> , Gln)	Phenylalanine (Phe)	



**Figure 11-4.** The biosynthesis of proline from glutamic acid in *E. coli*. Note the utilization of metabolic energy in the form of ATP in the initial step. (See text for details of synthetic steps.) Sections of the molecule reacting in each step are enclosed in color dashed lines.



**Figure 11-5.** The biosynthesis of methionine, threonine, and lysine: another example of metabolic energy utilization in the form of ATP in the interconversion of substances.

ability. All these processes, the interconversion and biosynthesis of chemical substances, require the expenditure of energy.

Consider the specific example of the synthesis of the amino acid proline by the bacterium *E. coli*. Glutamic acid is the initial reactant, itself formed from



the reductive amination of  $\alpha$ -ketoglutaric acid derived from the citric acid cycle. The steps involved are shown in Fig. 11-4. In the first step an acid group ( $-\text{COOH}$ ) is reduced to an aldehyde group ( $-\text{CHO}$ ). This requires two electrons from  $\text{NADPH}_2$  and energy from ATP. The aldehyde group then spontaneously reacts with the amino group ( $-\text{NH}_2$ ) on the same molecule, forming a ring. This step is followed by ring reduction to form proline.

Another example is the pathway for conversion of aspartic acid to lysine, methionine, and threonine. The conversion utilizes metabolic energy in the form of ATP, as shown in Fig. 11-5. These two examples serve to illustrate the expenditure of energy in the synthesis of amino acids. It may be added that the biosynthetic pathways for each of the 20 amino acids are well understood.

Just as amino acids are used by microorganisms to form proteins (see below for the process of protein synthesis), other low-molecular-weight organic precursors are polymerized to form other macromolecules. Nucleotides form nucleic acids (discussed later) and monosaccharides form polysaccharides. The precursors of lipids, especially the complex ones, include fatty acids, polyalcohols, amines, simple sugars, and even amino acids. It has been estimated that about 150 different small molecules are used to synthesize a new cell.

These small molecules are, in turn, synthesized from intermediates in the catabolic pathways of the microbes (Chap. 10). The most important of these intermediates are pyruvate, acetate, oxalacetate, succinate,  $\alpha$ -ketoglutarate, and the sugar-phosphates.

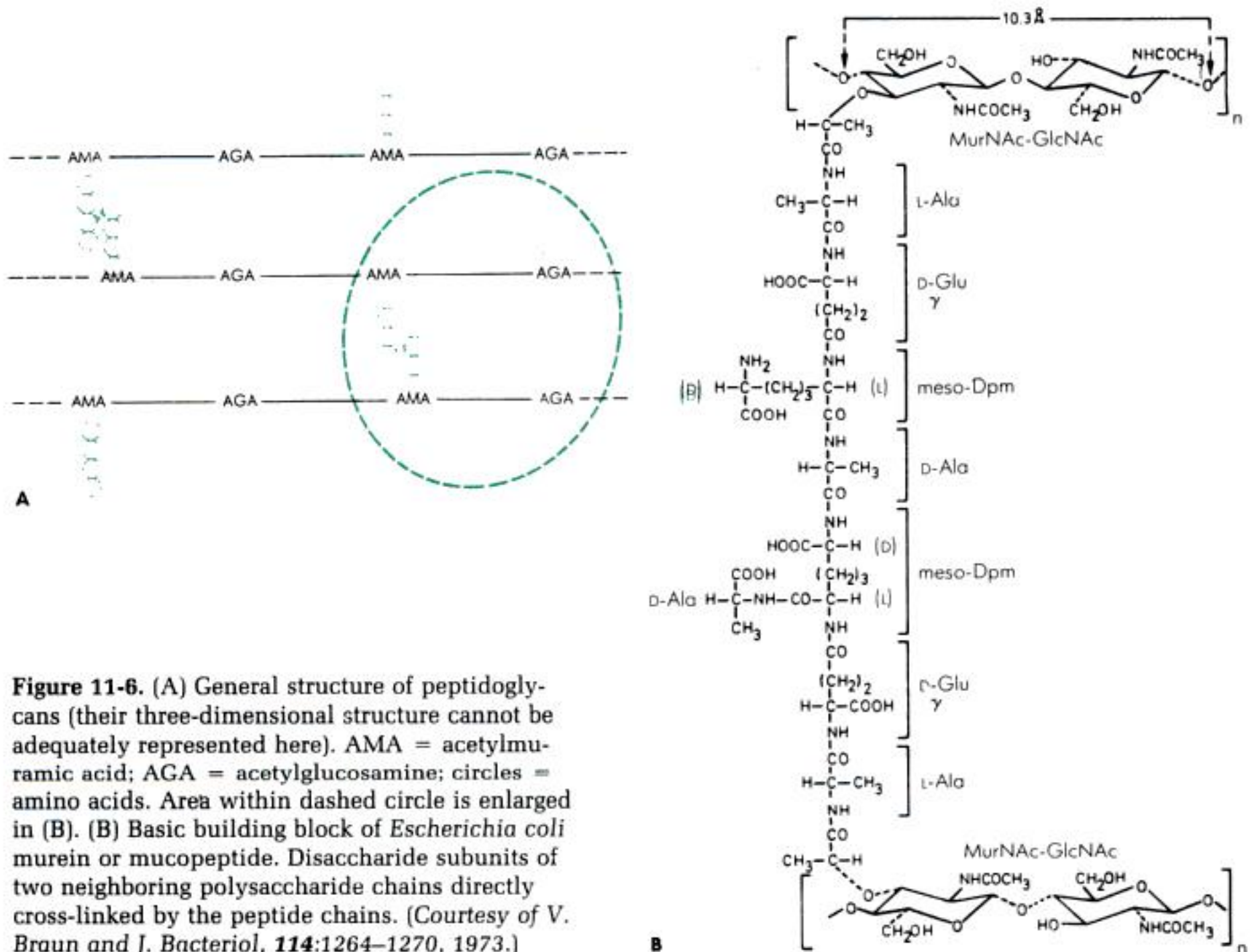
### Synthesis of Macromolecules: The Structure and Biosynthesis of a Cell-Wall Peptidoglycan

In all cells the major end products of biosynthesis are proteins and nucleic acids. However, there are other macromolecules peculiar to the procaryotes which require specialized biosynthetic processes. The utilization of energy in one of these processes is illustrated by the biosynthesis of bacterial cell-wall peptidoglycan. This particular biosynthetic process also serves as an example of how polymers are synthesized outside the membrane. Synthesis of cell-wall components is of interest because polymerization takes place outside the cell membrane by enzymes located on the membrane's outer surface.

### Structure of Peptidoglycan

As discussed in Chap. 5, the rigid portion of a bacterial cell wall is a polymeric structure known as a **murein**, **peptidoglycan**, or **cell wall peptidide**. The walls of Gram-positive bacteria contain a large proportion of peptidoglycan; those of Gram-negative bacteria have a much smaller proportion. Peptidoglycans vary in their chemical composition and structure from species to species, but there are basic similarities. Peptidoglycans are very large polymers composed of three kinds of building blocks: (1) **acetylglucosamine** (AGA or GlcNAc), (2) **acetylmuramic acid** (AMA or MurNAc), and (3) a **peptide** consisting of four or five amino acids of limited variety. Several of the amino acids exist in the D configuration, not usually found elsewhere in nature. A peptidoglycan can best be thought of as consisting of polysaccharide backbone chains composed of alternating units of AGA and AMA linked by  $\beta(1 \rightarrow 4)$  bonds, with the short peptide chains projecting from the AMA units. Many of these peptide chains are cross-linked with each other, imparting great rigidity to the total structure. Figure 11-6A illustrates the basic structure of peptidoglycans, and Fig. 11-6B shows a building block of the *E. coli* peptidoglycan. Some peptidoglycans differ in that





**Figure 11-6.** (A) General structure of peptidoglycans (their three-dimensional structure cannot be adequately represented here). AMA = acetylmuramic acid; AGA = acetylglucosamine; circles = amino acids. Area within dashed circle is enlarged in (B). (B) Basic building block of *Escherichia coli* murein or mucopeptide. Disaccharide subunits of two neighboring polysaccharide chains directly cross-linked by the peptide chains. (Courtesy of V. Braun and J. Bacteriol, **114**:1264–1270, 1973.)

the peptide chains may not be directly cross-linked to each other, being linked instead by another kind of peptide which forms a **bridge** between the terminal carboxyl group of one side chain with the free amino group of lysine or diaminopimelic acid (DPM or DAP) on the other side chain; e.g., in *Staphylococcus aureus* a bridge composed of five glycine molecules can link two muramic acid peptides together. This is shown in Fig. 11-7.

### Activation of a Peptidoglycan Precursor

*Escherichia coli* can synthesize cell-wall peptidoglycan when grown in a simple medium of glucose, ammonium sulfate, and mineral salts. One of the early steps in this synthesis is the formation of an activated derivative of AMA. This process, which is shown in Fig. 11-8, requires energy at several points and occurs in the cytoplasm. The activation of sugars, such as acetylglucosamine, by the attachment of a uridine diphosphate (UDP) to form a sugar-UDP precursor is not peculiar to AMA but is a general method involved in the biosynthesis of many kinds of polysaccharides.





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stranded DNA is always 1:1. That is, the amount of purines is equal to the amount of pyrimidines.

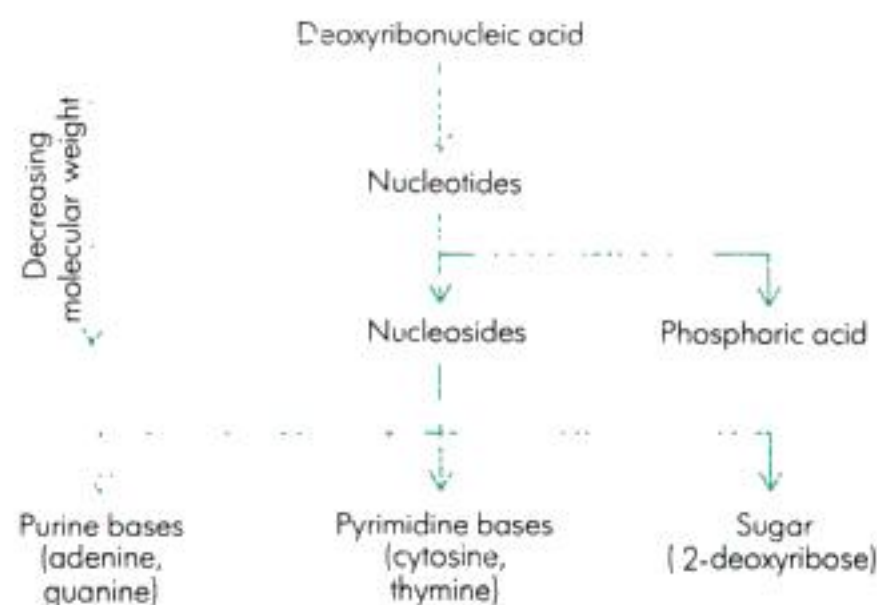
The DNA of each species shows a characteristic composition that is not affected by age, growth, conditions, environmental changes, etc. The molar ratio  $\frac{[A] + [T]}{[G] + [C]}$  indicates a characteristic composition of DNA of each species. For example, in humans this value is 1.52; in sheep, 1.36; in wheat germ, 1.19. Indeed, these values can even be used at the species level in bacteria for identification or taxonomic grouping: *Escherichia coli*, 0.93; *Staphylococcus aureus*, 1.50; *Clostridium perfringens*, 2.70; *Micrococcus lutea*, 0.35. (However, in bacterial taxonomy, the differences in base composition between species are more commonly expressed as  $[G + C]$  percent of total bases. This was explained in Chap. 3.)

The failure to find a 1:1 ratio between adenine and thymine or guanine and cytosine in certain viruses led to the discovery of single-stranded DNA in these organisms. For example, phage  $\phi$ X174 contains a single strand of DNA in a ring form. However, these cases are rare exceptions to the general occurrence of double-stranded DNA.

The complementary base pairs hold the two strands of the DNA helix together by hydrogen bonding. As shown in Figs. 11-13 and 11-15, there are two hydrogen bonds formed between each A-T pair, whereas there are three hydrogen bonds formed between each G-C pair. The complementarity of the purines and pyrimidines means that the sequence of bases on one strand dictates the sequence on the other strand. This is of critical importance in the synthesis, or **replication**, of new strands of DNA during cell division. A consequence of the formation of the A-T and G-C pairs is that the two strands of the DNA helix are said to be **antiparallel**, or to have opposite polarities. This means that each strand runs in opposite directions so that one is terminated by a free 3'-hydroxyl group and the other by a 5'-phosphate group (Fig. 11-15), where 3' and 5' refer to the numbering of the carbon atoms of the deoxyribose molecule. If you examine the nucleotides in Fig. 11-15, you will find that on the left strand the phosphate on the fifth carbon (5') of the sugar points up. On the right strand the phosphate on the fifth carbon points down. Each strand keeps the same polarity as it winds around the molecule.

The relationship of DNA to its low-molecular-weight components is shown

**Figure 11-16.** Breakdown of deoxyribonucleic acid into lower-molecular-weight components. DNA is made up of nucleotides which, when you remove phosphoric acid, yield nucleosides. Nucleosides can be broken down into bases and sugar (2-deoxyribose).



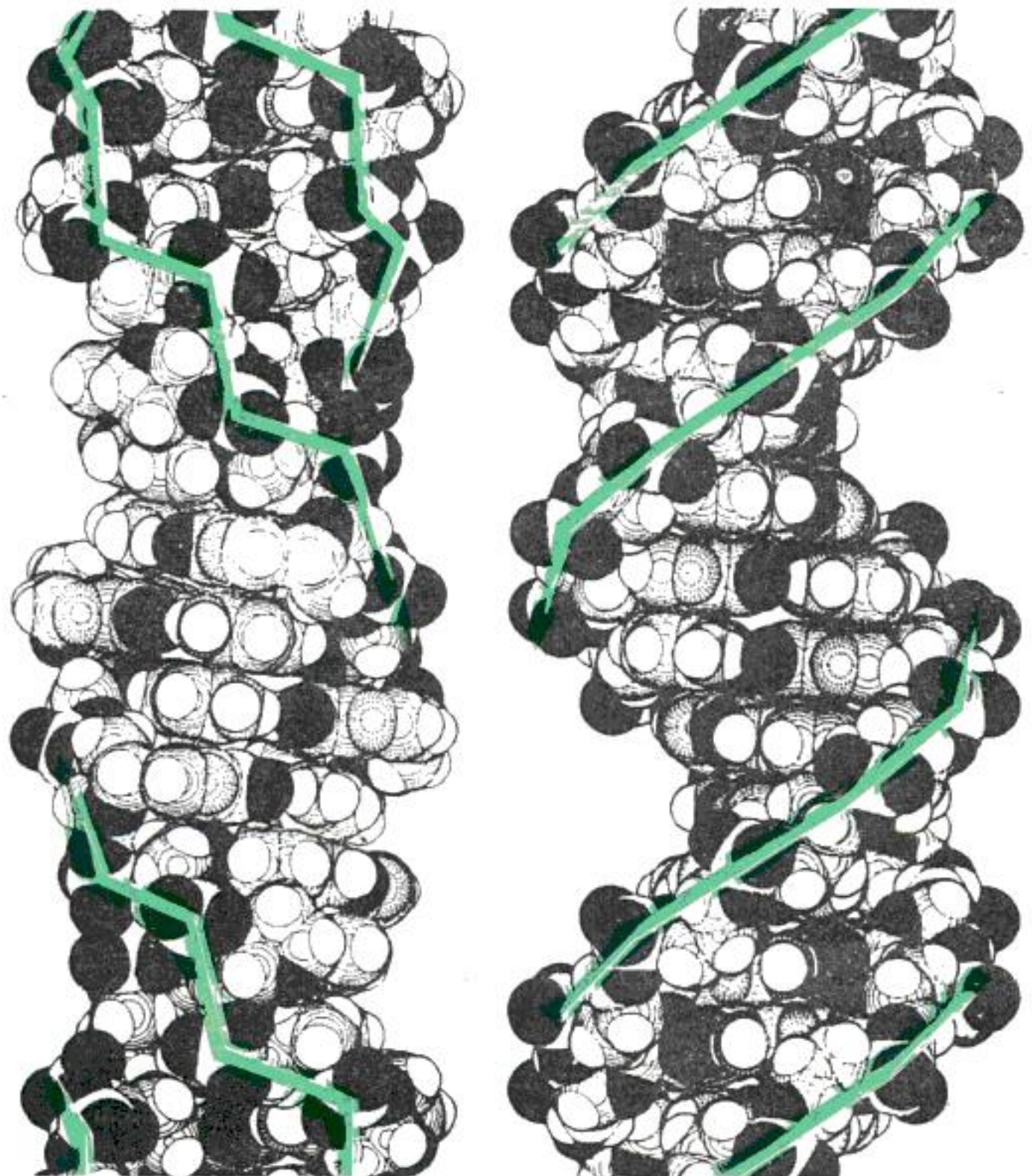


in Fig. 11-16. The removal of the phosphate group from the nucleotide yields a **nucleoside** consisting of a pentose sugar linked to a heterocyclic base.

The helical structure of the DNA molecule proposed by Watson and Crick is a right-handed, double-stranded one. This double helix is right-handed because the turns run clockwise looking along the helical axis. It represents what is known as the B form of DNA. But recent evidence indicates that DNA may be able to exist in other types of double-helical structures. There are A and C forms of the DNA helix; they differ from the B form in several features, such as the numbers of base pairs per turn, the vertical rise per base pair, or the helical diameter.

However, the Z form exhibits the most contrast with the other forms because it is a left-handed helix (sinistral DNA). It has the most base pairs per turn. Its name is taken from the anticlockwise zigzag path that the sugar-phosphate backbone follows along the helix. The structures of Z-DNA and B-DNA are compared in Fig. 11-17.

**Figure 11-17.** Left-handed (Z-form) and right-handed (B form) DNA models. The superimposed line traces the sugar-phosphate backbone; in the B form it is a continuous helix, while in the Z form the backbone zig-zags. (Reproduced from *Mosaic*, 14:2, 1983.)



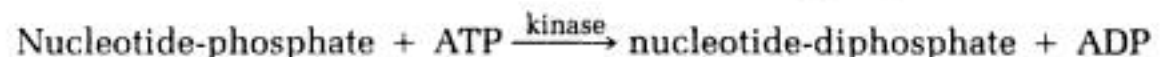
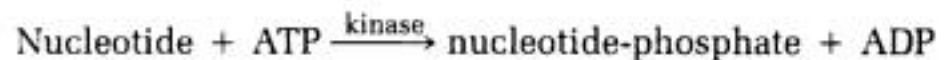


Z-DNA has been found in a variety of living organisms, including rats, rabbits, several species of plants, and protozoa. It appears that wherever there is regulation of gene action there is found Z-DNA. That is, Z-DNA seems to be important for biological development and control.

The other naturally occurring nucleic acid is ribonucleic acid (RNA). It plays a fundamental role by making it possible for the sequence of chemical groups in DNA to dictate the sequence of amino acids in proteins. It differs from DNA in these respects:

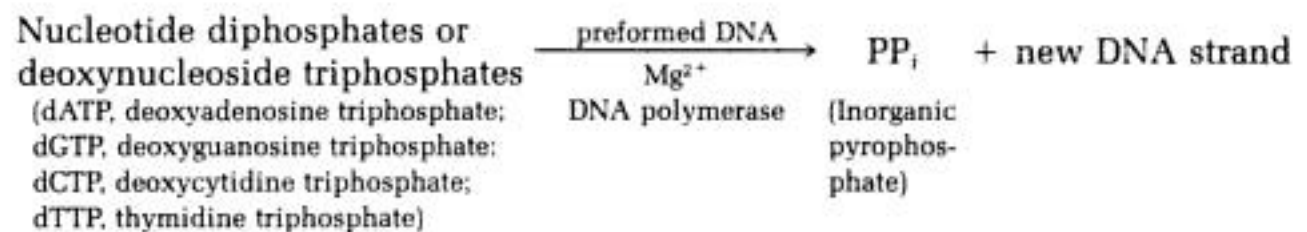
- 1 The sugar component of the nucleotides which make up RNA is ribose, instead of deoxyribose as in DNA. Ribose is similar to deoxyribose except for the presence of a hydroxyl group at the number-2 (2') carbon atom (see Fig. 11-14).
- 2 The pyrimidine nitrogenous base uracil, instead of thymine, is found in the nucleotides that make up RNA (see Fig. 11-14).
- 3 It is usually single-stranded. The single strands can bend themselves backward and base-pair to form three-dimensional structures.
- 4 RNA is degraded to its nucleotides much more easily than DNA.

Before the polynucleotide chains of DNA can be synthesized by bacteria (or any other organisms), an intracellular pool of nucleotides must be available. In some bacteria, these nucleotides must be supplied preformed in the medium, but in bacteria with relatively simple nutritional requirements, this reservoir of nucleotides can be synthesized from glucose, ammonium sulfate, and some minerals. The conversion of simple nutrients into nucleotides for DNA synthesis involves a complex series of enzymatically catalyzed reactions, several of which require energy in the form of ATP. Some of these reactions form activated nucleotides as direct precursors for synthesis of the polynucleotide chains of double stranded DNA:



As seen in the above equations, energy in the form of ATP is utilized.

The overall chemical reaction is



The new DNA strand is complementary to the preformed or template DNA strand.

Prior to cell division, the two DNA chains of a DNA molecule separate by unwinding, and each serves as a template for the synthesis of a new complementary chain, thus forming two new helices, each exactly like the original. Each half of the dividing cell then receives one of these helices. This type of



## Replication of the DNA Molecule

duplication in which one polynucleotide chain acts as a template to direct the synthesis of a new chain complementary to itself is termed **semiconservative replication**; it results in two daughter helices, each containing one old template strand and one new complementary strand. In other words, only one of the old strands is conserved in each daughter helix.

Bacteria are almost always **haploid**, which means that their chromosomes are unpaired. In contrast, most eucaryotic cells (except for gametes) are **diploid**; they have paired homologous chromosomes which may be **heterozygous** (carry different alleles or genes occupying the same relative locus on homologous chromosomes). All bacteria studied to date have their genetic loci in a single linkage group; that is, they have a single chromosome per genome.

The chromosome of a typical bacterium is a circular double-stranded DNA molecule; that is, the double helix for a complete genome has no free ends. It has an approximate molecular weight of  $2.5 \times 10^9$  daltons (a dalton is equal to the mass of one hydrogen atom) and has about  $4 \times 10^6$  base pairs. The circular chromosome is further twisted on itself in the bacterial cell to form a supercoil. (This is also the case in many viruses.) If, instead, the chromosome were extended linearly, it would measure approximately 1250  $\mu\text{m}$  (1.25 mm), which is several hundred times longer than the bacterial cell that contains it. A circular form is typical of the DNA molecules of procaryotic microorganisms, of viruses, and of organelles in eucaryotic organisms. However, not all DNA molecules are circular; the chromosomes of eucaryotic organisms and of many viruses consist of linear DNA molecules.

There are three general methods of replication of the DNA molecule.

### $\theta$ (Theta) Mode

The replication of a circular DNA molecule is initiated at a certain point called the **origin**, which is specific for each bacterial species. Replication proceeds in two directions around the chromosome, leading to the formation of a "bubble," which increases in size as replication proceeds. This mode is called **theta** because intermediate structures resemble the Greek letter  $\theta$  (see Fig. 11-18). In this process, a circular parental chromosome is replicated to two circular daughter chromosomes, in each of which one strand of the parental DNA molecule is conserved and a complementary strand is newly synthesized. Figure 11-18 illustrates this  $\theta$  mode of replication of the DNA molecule.

### $\sigma$ (Sigma) or "Rolling Circle" Mode

Replication begins with the cleavage of a phosphodiester bond in one strand of the circular DNA molecule to produce a nick with 3'-OH and 5'-PO<sub>4</sub> ends on that strand. The complementary circular strand then serves as a template for the synthesis of a new strand, which is covalently linked to the 3'-OH end of the nicked parental strand. As this strand grows at the 3'-OH end, the 5'-PO<sub>4</sub> end of the same strand is displaced to form a "tail" on the circle. As replication proceeds, a circular parental molecule is converted to two daughter molecules, one circular and the other linear. This mode is called **sigma** because intermediate structures have the Greek letter  $\sigma$  conformation (see Fig. 11-19). The sigma mode of DNA molecule replication is carried out by some bacteriophages, such as  $\lambda$  and  $\phi\text{X174}$  whose progeny viral DNA is linear; by bacteria involved in sexual conjugation; and by certain eucaryotes during oogenesis.



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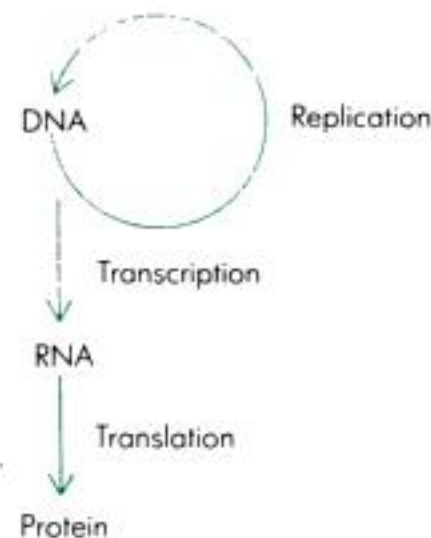
Initiation of DNA replication requires a **primer**, a short sequence of RNA that is synthesized by RNA polymerase and is complementary to the DNA which serves as a template. (These RNA primers are used because RNA polymerase, unlike DNA polymerase, requires only template but not primer. "Polymerase" implies a polymerization action.) The RNA primer's 3'-OH end serves for the addition of nucleotide diphosphates by DNA polymerase III. DNA polymerase I then removes the RNA primer with its 5'-nuclease activity; simultaneously it fills in the gap with DNA via its 3'-polymerase activity. This DNA polymerase I uses the 3'-OH end of the preceding DNA fragment as its primer. Thus as it removes the RNA, it extends or elongates the preceding DNA molecule that served as its primer. When the entire RNA is removed, a single-stranded nick remains between the two DNA fragments and is sealed by DNA ligase, a DNA-joining enzyme. (DNA polymerase II is found also in *E. coli*, but, as yet, no specific function has been ascribed to it.)

## TRANSCRIPTION AND TRANSLATION OF GENETIC INFORMATION

We have examined how DNA is replicated as a cell grows and before it divides into two cells. One of these DNA copies is then transmitted to the progeny or sister cell. In this way genetic information programmed in the genes is passed on from an organism to its offspring. Thus, DNA, or the genes, is a very important molecule in the cell. It is carefully guarded from damage, and it is repaired when damage is detected. (On the other hand, RNA and proteins are subject to degradation and replacement rather than to repair; that is, they undergo **turn-over**.) We will now see how genetic information is coded in the DNA, and how the genetic code is deciphered and used in the production of proteins. All these processes constitute what is known as the **central dogma** of molecular genetics, which may be described in three steps. The first is DNA **replication** (which we have just studied); the second is **transcription**, the process in which the genetic message on DNA is transcribed to RNA; and the third is **translation**, in which the genetic message coded by RNA is translated by the ribosomes into a protein structure. The outline of the central dogma is shown in Fig. 11-22.

### The Building Blocks of Proteins

Just as nucleotides are building blocks of DNA, amino acids are building blocks of proteins. However, DNA consists of only four kinds of nucleotides, whereas proteins consist of about 20 kinds of amino acids. Microorganisms differ widely in their ability to synthesize amino acids. For example, *E. coli* can synthesize



**Figure 11-22.** Processes in the central dogma of molecular genetics.



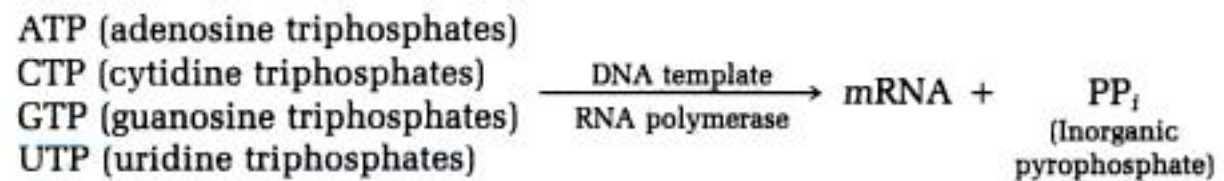




pattern constitutes the **secondary structure** of the protein. The amino acid side chains or groups of a polypeptide (e.g., cysteine residues) contribute further to the folding process by forces of attraction and repulsion; this process gives a protein its **tertiary structure**. In Fig. 11-23, the tertiary structure of RNAase is seen to be contributed by four disulfide bridges. As may be observed, RNAase is a single polypeptide chain of 124 amino acid residues; it is folded, bent, and twisted into a globular shape in its active form. Finally, the overall shape resulting from the interaction of two or more polypeptide chains constitutes the **quaternary structure** of a protein.

## Transcription

Protein synthesis takes place on the ribosomes, which are large RNA-protein particles in the cytoplasm of the bacterial cell. (In eucaryotic cells the ribosomes are attached to the endoplasmic reticulum.) **Ribosomal RNA (rRNA)** constitutes about 90 percent of the total cellular RNA. Before protein synthesis can proceed, the coding of DNA must first be transferred to a substance that passes information from the DNA in the nuclear region to the ribosomes in the cytoplasm. This substance is known as **messenger RNA (mRNA)**. The process or step in which a single-stranded mRNA is synthesized complementary to one DNA strand is called transcription. The synthesis of the polynucleotide chain of mRNA is catalyzed by the enzyme RNA polymerase. Just as activated deoxyribonucleotides are required in DNA synthesis, activated ribonucleotides are required as substrates for this enzyme:

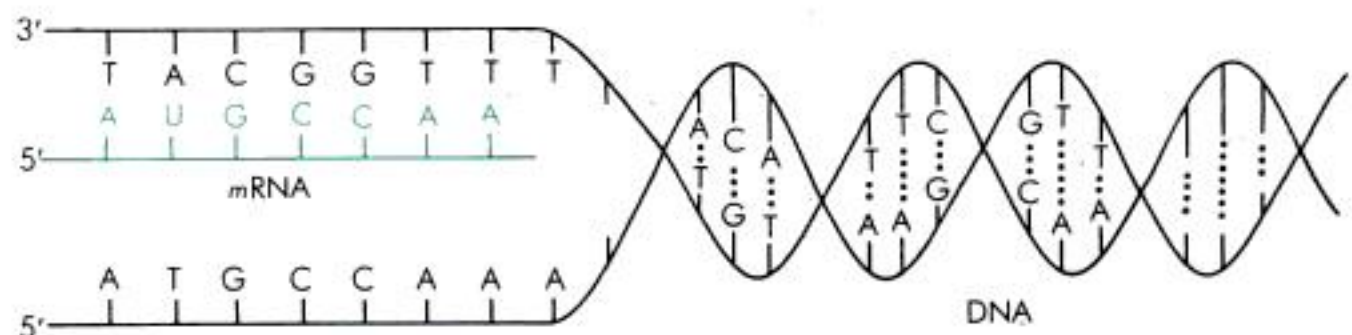


The synthesis of RNA is different from that of DNA in the following features:

- 1 Only one of the two strands of any given segment of DNA serves as the template.
- 2 Only specific, relatively short lengths of DNA are transcribed; i.e., an RNA chain is a transcript of a short section of DNA.

Transcription is the first step in gene expression. This process, as shown in Fig. 11-24, involves separation of the two DNA strands, one of which serves as a template for the synthesis of a complementary strand of mRNA by DNA-dependent RNA polymerase. When a short RNA chain is completed, the DNA double helix closes again. The strand of DNA selected for transcription in a given segment is called the "sense" strand and contains a specific **initiation site**, which is a regulatory sequence of DNA nucleotides called the **promoter region**. (The other strand may be the "sense" strand for another group of genes

**Figure 11-24.** Transcription of DNA by DNA-dependent RNA polymerase. The chain of mRNA is being synthesized from the information in DNA. Note how complementarity is maintained. For example, where there is a G in DNA, a C is inserted in mRNA, and where there is an A in DNA, a U is inserted in mRNA. The mRNA will serve as a pattern for protein synthesis. The growth of a mRNA chain proceeds in a 5' → 3' direction in a manner similar to DNA replication. (A, adenine; T, thymine; C, cytosine; G, guanine; U, uracil.)





in another segment.) In bacteria, the initiation of RNA polymerase activity at this site is due to an initiation factor called the **sigma factor**, which is a component of the enzyme. Termination of mRNA synthesis is also at specific regulatory sequences of DNA nucleotides along the DNA molecule which are recognized by the RNA polymerase. Furthermore, a tetrameric protein factor called the **rho** factor binds to RNA polymerase and promotes its termination. When transcription has been completed, rho dissociates from the RNA polymerase-DNA complex.

## Translation

Translation, the next step in gene expression, is the process in which the genetic information now present in the mRNA molecule directs protein synthesis.

When the four different bases of the nucleotides of mRNA are arranged in sequences of three, each base triplet, called a **codon**, is capable of specifying a particular amino acid. Since there are four different bases, the number of sequences of three of them is  $4^3$ , or 64. These base triplets, each of which specifies a particular amino acid, constitute the genetic code (Table 11-2). The code is probably universal for all species of living organisms.

How is this code translated? Using Table 11-2, suppose the base sequence of mRNA is

CUUAGAAAUUUAGUGGGACUUCU

The translation of this code into amino acids in a polypeptide chain, at a ribosome, would be

Leu-Arg-Lys-Phe-Ser-Gly-Thr-Ser

**Table 11-2.** The Genetic Code for the Base Triplets of mRNA and the Amino Acids They Code for\*

First Base	Second Base				Third Base
	U	C	A	G	
U	UUU } Phenylalanine	UCU } Serine	UAU } Tyrosine	UGU } Cysteine	U
	UUC } Phenylalanine	UCC } Serine	UAC } Tyrosine	UGC } Cysteine	C
	UUA } Leucine	UCA } Serine	UAA "Ochre"	UGA "Umber"	A
	UUG } Leucine	UCG } Serine	UAG "Amber"	UGG Tryptophan	G
C	CUU } Leucine	CCU } Proline	CAU } Histidine	CGU } Arginine	U
	CUC } Leucine	CCC } Proline	CAC } Histidine	CGC } Arginine	C
	CUA } Leucine	CCA } Proline	CAA } Glutamine	CGA } Arginine	A
	CUG } Leucine	CCG } Proline	CAG } Glutamine	CGG } Arginine	G
A	AUU } Isoleucine	ACU } Threonine	AAU } Asparagine	AGU } Serine	U
	AUC } Isoleucine	ACC } Threonine	AAC } Asparagine	AGC } Serine	C
	AUA } Isoleucine	ACA } Threonine	AAA } Lysine	AGA } Arginine	A
	AUG Methionine	ACG } Threonine	AAG } Lysine	AGG } Arginine	G
G	GUU } Valine	GCU } Alanine	GAU } Aspartic acid	GGU } Glycine	U
	GUC } Valine	GCC } Alanine	GAA } Glutamic acid	GGC } Glycine	C
	GUA } Valine	GCA } Alanine	GAG } Glutamic acid	GGA } Glycine	A
	GUG } Valine	GCG } Alanine		GGG } Glycine	G

\* The codons read in the 5' to 3' direction (left to right) on the mRNA. Codons UAA (ochre), UAG (amber), and UGA (umber) cause termination of synthesis of a protein chain. AUG and GUG are chain-initiating codons. Note that the same amino acid may be coded for by more than one codon (such a code is called degenerate). But no codon codes for more than one amino acid.



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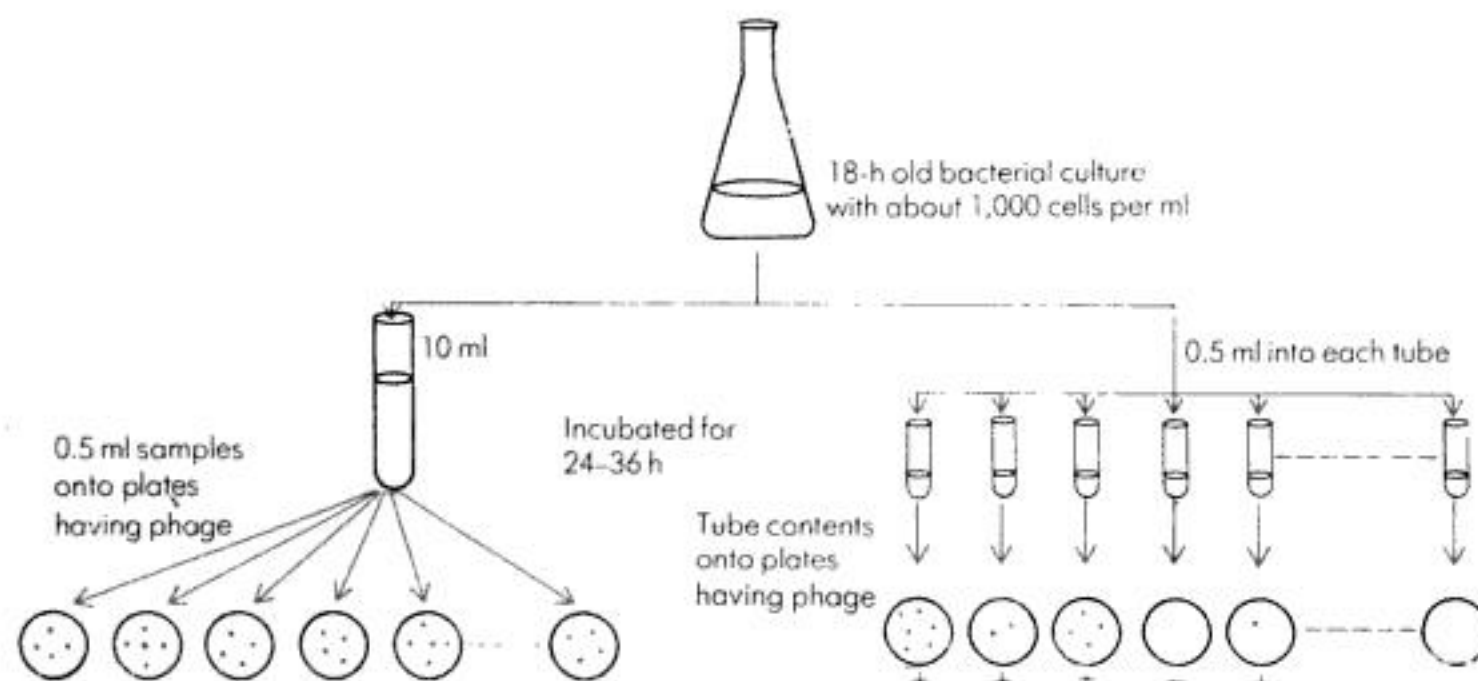


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**Figure 12-4.** The fluctuation test was performed essentially as follows. A series of tubes containing 0.5 ml of cells was incubated without phage until a certain population size was reached. The cultures were then exposed to phage by pouring the contents of each tube into an agar plate containing phage. The number of phage-resistant mutants in each tube was thus determined. The colony counts from such a series of similar cultures were then compared with the results of a series of samples taken from one culture started with a similar density of cells per milliliter and allowed to reach a similar population number per milliliter. The results showed that resistant bacteria arise spontaneously prior to the exposure to phage since a series of similar cultures yielded results different from those obtained with a series of samples from one culture. (See text for further explanation.)

more than physiological adaptations or phenotypic changes. That is, they refused to believe that bacteria have stable hereditary systems and could undergo permanent changes or mutations and assumed that any changes in the characteristics of a culture were simply due to environmental influences. However, Max Delbrück and Salvador Luria believed that bacteria have stable hereditary mechanisms, and in 1943 they performed an elegant experiment that proved the point.

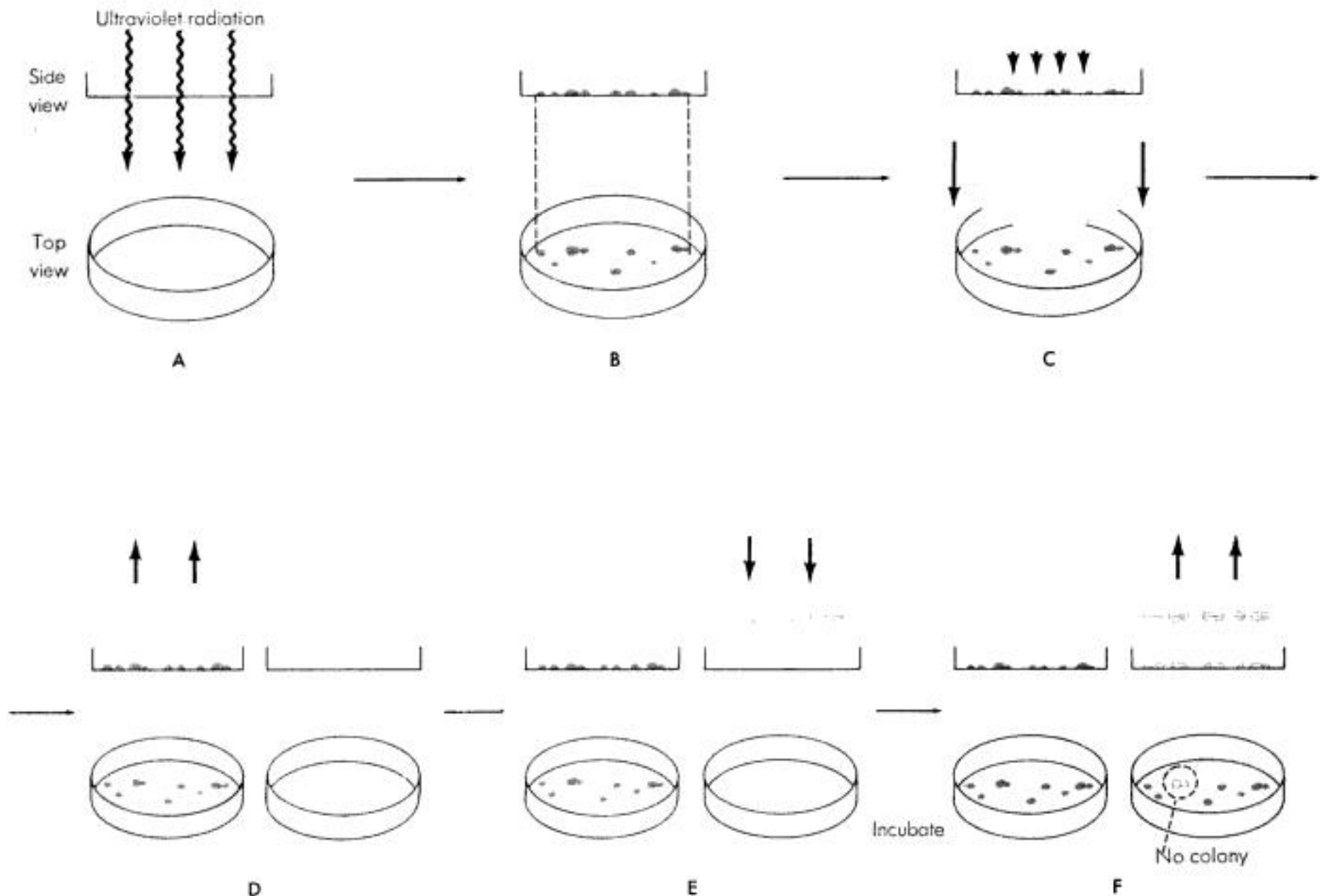
Bacterial viruses called bacteriophages, or simply phages, are capable of killing bacteria. When susceptible bacteria are exposed to a phage, some of the bacterial cells survive, and they and their descendants are resistant to the phage. Some microbiologists assumed that these cells were modified by their contact with the phage so they became resistant by physiological adaptation. But Luria and Delbrück believed that phage-resistant bacteria were the result of mutations that occurred before the bacteria came into contact with the phages.

Let us suppose that resistant cells are the result of contact with phages; then if we set up a large number of identical bacterial cultures and expose them all to identical batches of phage, approximately the same number of resistant cells should appear in all cultures. However, if the resistant cells are really the result of mutations and since mutations occur entirely at random, then when we grow many identical cultures and expose them all to identical batches of phage, we should find a great fluctuation in the numbers of resistant cells. In some cultures, there may be no mutations and therefore no resistant cells, but in other cultures a mutation might have occurred very early, so that nearly all the cells are resistant (Fig. 12-3).

Thus Luria and Delbrück predicted that physiological adaptation to the phage by the bacteria should give about the same number of resistant bacteria (within sampling error) in each culture. But if phage resistance was due to mutations, preexisting and merely selected by the addition of phage, the number of resistant bacteria should fluctuate widely in each culture. Luria and Delbrück found a much greater fluctuation in numbers than could be accounted for by physiological adaptation, and thus they proved statistically by their fluctuation test that phage resistance was really the result of mutation (see Fig. 12-4).

Shortly after, in 1952, more direct proof of preexisting mutants was provided

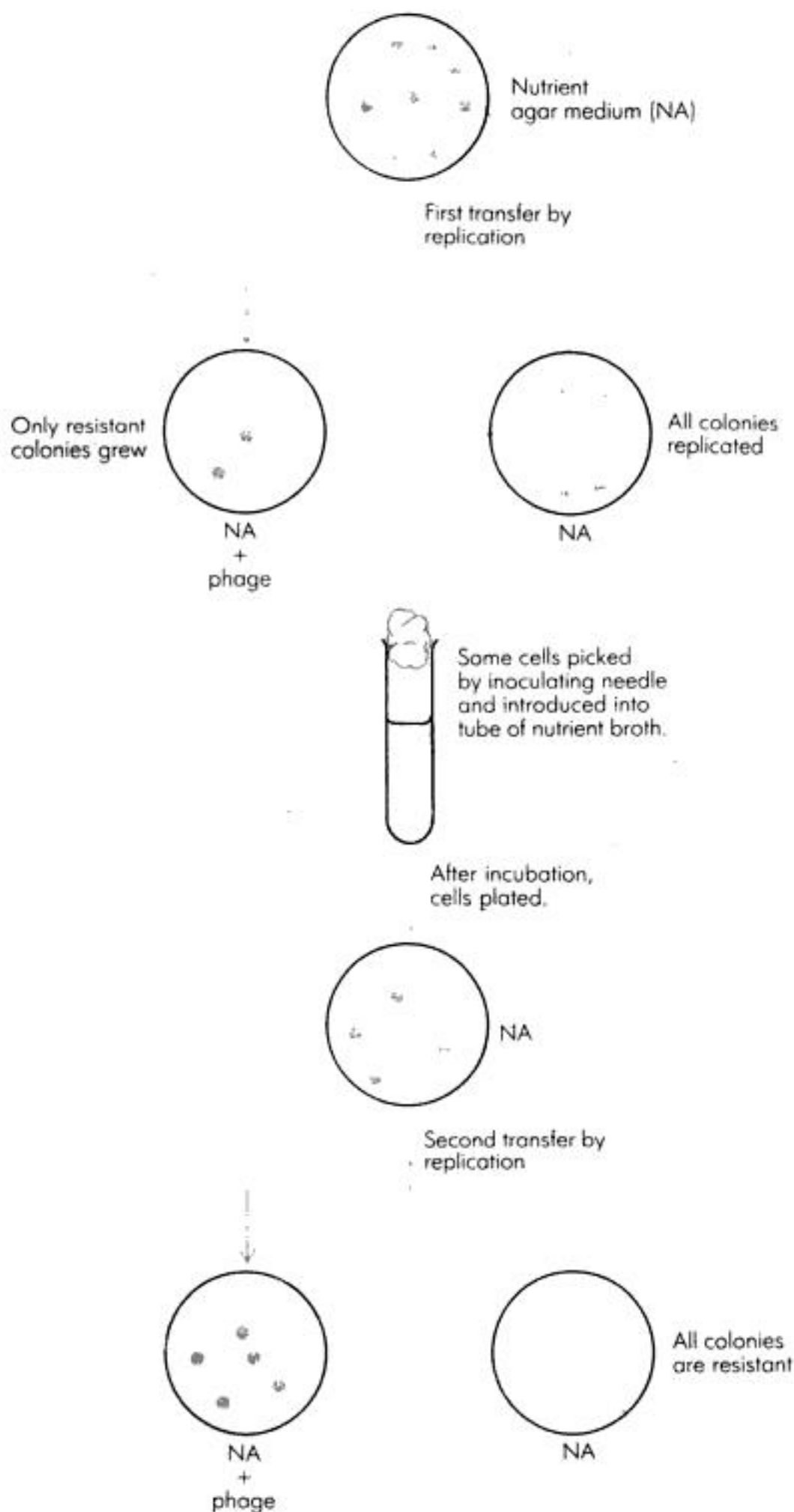
by Joshua and Esther Lederberg. They introduced the replica plating technique (shown in Fig. 12-5) and provided a direct method for demonstrating the un-directed spontaneous origin of bacterial mutants; i.e., the mutants occurred independently of any selective agent or environment. The procedure made it



**Figure 12-5.** Replica plating is used for isolating nutritional mutants of *Escherichia coli*. (A) Bacterial suspension placed in open half of Petri dish and exposed to mutagenic agent, such as ultraviolet radiation. (B) Sample from (A) plated on surface of a "complete" medium such as nutrient agar. The plate is incubated; after incubation, the exact position of colonies on the plate is noted. (C) A sterile replica plating unit is gently pressed to the surface of plate (B), then raised (D), and then pressed to the surface of a plate of "minimal" agar medium (E). The positioning of the replica plating unit on the minimal agar must be precise, so that colony locations will be comparable on each of the two plates. The plates will be replicas of one another. The minimal agar in the plate in (E) consists of inorganic salts and glucose, nutrients which normally permit growth of *E. coli*. After incubation (F), colonies appear on the new plate at most, but not all, of the positions corresponding to locations of colonies on the original plate. It may be assumed that the organisms that failed to develop are nutritional mutants; that is, they are not able to grow on an inorganic salts-glucose medium, a characteristic which they originally possessed.

practical to examine large numbers of *clones* (populations of cells descending from a single cell) for a particular characteristic. By using sufficiently large samples, one could, for example, demonstrate the occurrence of phage-resistant mutants in a culture which was known to be phage-sensitive. The mutant types developed and could be located on an agar-plate culture which had not en-

**Figure 12-6.** Simplified representation of the experiment of the Lederbergs showing the spontaneous nature of mutation in bacteria. The drawing shows that isolation of a pure colony of phage-resistant bacteria from a medium is possible without prior exposure to the virus.





countered phage previously. Similarly, the spontaneous appearance of antibiotic-resistant strains could be demonstrated without previous exposure of the culture to the antibiotic. As Fig. 12-5 shows, replica plating can also be used for isolating nutritional mutants. In essence the technique provides a practical means for finding the one cell in a million (more or less) which has mutated.

Thus the Lederbergs were able to isolate pure colonies of resistant *E. coli* mutants from many colonies on a medium plate that had never been exposed to lytic phage. This experiment demonstrated that mutation against the phage had its origin in spontaneous mutation. The growth of resistant colonies on replica plates arose from cells that were already present and were already resistant on the original nonselective plate prior to exposure to the selective agent, such as a lytic phage. Figure 12-6 shows a simplified representation of the experiment performed by the Lederbergs. Since then many other types of mutations have been found in bacteria, and it is now firmly established that bacteria have a hereditary system just like higher organisms.

## Types of Mutations

At the molecular level there are several ways in which changes in the purine-pyrimidine base sequence of a gene can occur, resulting in mutation. Two common types are **point mutations** and **frameshift mutations**.

### Point Mutations

Point mutations occur as a result of the substitution of one nucleotide for another in the specific nucleotide sequence of a gene. The substitution of one purine for another purine or one pyrimidine for another pyrimidine is termed a **transition** type of point mutation. A **transversion** is the replacement of a purine by a pyrimidine, or vice versa. This **base-pair substitution** may result in one of three kinds of mutations affecting the translation process:

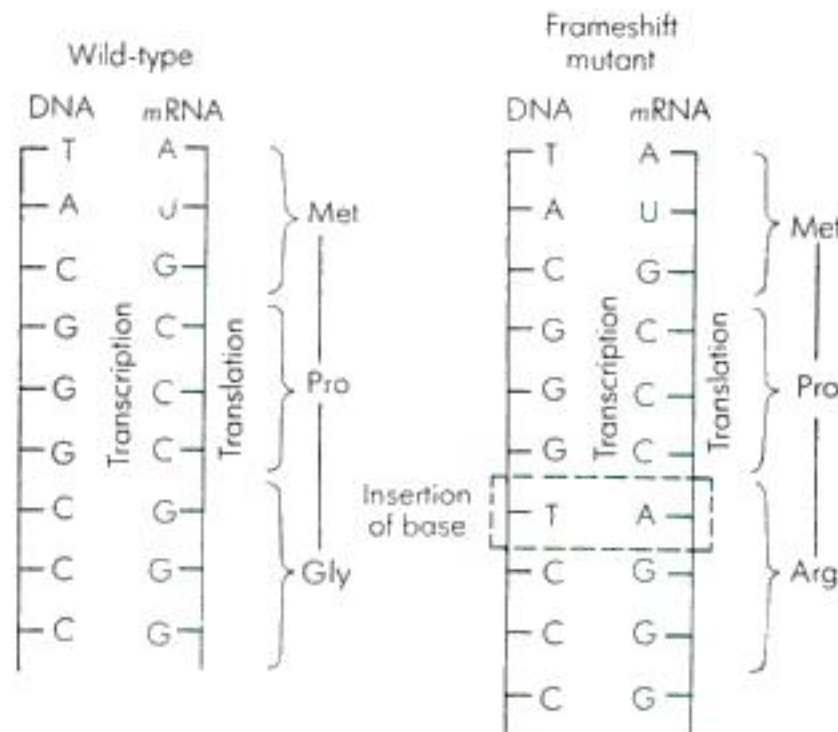
- 1 The altered gene triplet produces a codon in the mRNA which specifies an amino acid different from the one present in the normal protein. This mutation is called a **missense mutation**. Such a protein may be functionally inactive or less active than the normal one. A good example of a missense mutation in humans is the disease sickle cell anemia. A single base substitution in the codon for the sixth amino acid of normal hemoglobin A changes the sixth amino acid from glutamic acid to valine, thus forming the characteristic hemoglobin S of sickle cell anemia. That is, GAG, which codes for glutamic acid, has changed to GUG for valine. Under low oxygen concentration the altered hemoglobin S molecules stack into crystals, giving the red blood cells a sickle shape.
- 2 The altered gene triplet produces a chain terminating codon in mRNA, resulting in premature termination of protein formation during translation. This is called a **nonsense mutation**. The result is an incomplete polypeptide which is nonfunctional.
- 3 The altered gene triplet produces a mRNA codon which specifies the same amino acid because the codon resulting from mutation is a synonym for the original codon. This is a **neutral mutation**.

### Frameshift Mutations

These mutations result from an *addition* or *loss* of one or more nucleotides in a gene and are termed **insertion** or **deletion** mutations, respectively. This results in a shift of the reading frame. We saw earlier that during protein synthesis the reading of the genetic code starts from one end of the protein template, mRNA,



**Figure 12-7.** Frameshift mutation, as a result of insertion of a nucleotide in a gene. Insertion of a nucleotide in a gene results in the transcription of an additional nucleotide in mRNA. This results in a frameshift when codons are read during translation, so all codons following the insertion are altered and all amino acids coded for are changed. A frameshift mutation as a result of deletion of a nucleotide would have essentially the same effect.



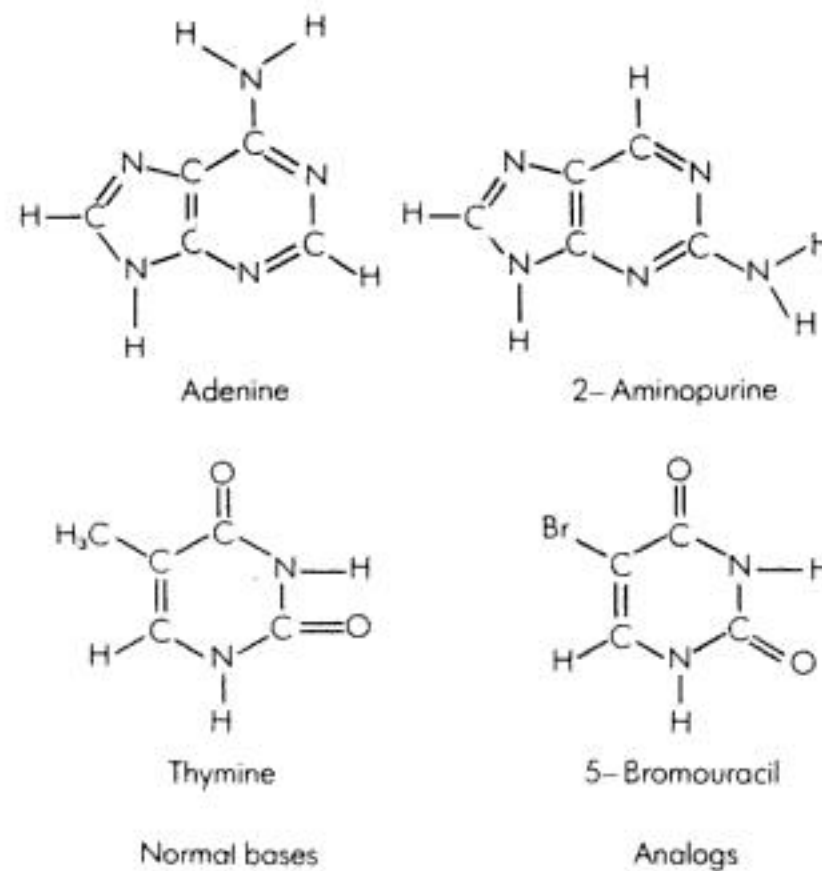
and is read in consecutive blocks of three bases. Frameshift mutations, therefore, generally lead to nonfunctional proteins, because an entirely new sequence of amino acids is synthesized from a frameshift reading of the nucleotide sequences of mRNA (which was transcribed from a mutation in the DNA of the cell). This type of mutation is illustrated in Fig. 12-7.

## How Mutations Occur

Mutations most commonly occur during DNA replication. Some mutations occur as the result of damages inflicted by ultraviolet (UV) light or x-rays. Since these agents are an inescapable part of the environment (for example, UV light is a component of sunlight), they probably account for many spontaneous mutations. However, mutation rates can be increased substantially by deliberately exposing a culture to such radiation. Any agent that increases the mutation rate is called a **mutagen**. Mutations obtained by use of a mutagen are said to be **induced**, rather than spontaneous, though they may differ only in frequency, not in kind. For example, UV light causes mutation under both natural and laboratory conditions. The number of mutants obtained by laboratory conditions is much higher, however, because of the high dosage of UV light used.

The major effect of UV light is to cause the formation of **dimers** by cross-linking between adjacent pyrimidine, especially thymine, residues in DNA. These cross-linked residues disrupt the normal process of replication by preventing the various polymerases from functioning. When x-rays interact with DNA, the result is usually a break in the phosphodiester backbone of the nucleic acid.

The most revealing findings about mutation in recent years have come from studies on the mutagenic effects of various chemicals. There are three main types of mutagenic chemicals. The first consists of compounds that can react chemically with DNA. Since specificity of DNA replication depends upon purine-pyrimidine bonding, which results from hydrogen bonding between the amino and hydroxyl groups of the purines and pyrimidines, chemical modification of these amino and hydroxyl groups can cause mutation. Nitrous acid, which can remove amino groups from purines and pyrimidines, is such a



**Figure 12-8.** Two normal DNA bases and two base analog mutagens. 2-Aminopurine is an analog of adenine and can pair with thymine or cytosine. 5-Bromouracil is an analog of thymine and can pair with adenine or guanine. A color box highlights the part of the analog which differs from the normal base.

mutagen. The second type of mutagenic chemicals consists of *base analogs*. These are chemicals sufficiently similar in structure to normal DNA bases to be substituted for them during DNA replication (Fig. 12-8). Although similar in structure, base analogs do not have the same hydrogen-bonding properties as the normal bases. They can therefore introduce errors in replication which result in mutation. A third type of mutagenic chemicals is *intercalating agents*. These are flat molecules that can intercalate (slip in) between base pairs in the central stack of the DNA helix. By this means they distort the structures and cause subsequent replication errors. Examples of such agents are acridine orange, proflavin, and nitrogen mustards.

Recently, it was shown that mutations can occur because of transposons. *Transposons* are units of DNA which move from one DNA molecule to another, inserting themselves nearly at random. They are also capable of causing DNA rearrangements such as deletions or inversions. For example, one such transposon is from the bacterial virus called Mu, which may be considered a mutagen.

We have said that DNA damage can occur by UV radiation, x-rays, and certain chemicals. Fortunately, cells contain specific enzymes which can repair damaged DNA. In this way, some affected cells can continue to function normally.

Many kinds of bacterial cells and yeasts have been shown to possess an efficient photoreactivating mechanism for repairing damage caused by UV radiation. This *photoreactivation* occurs when cells exposed to lethal doses of UV light are immediately exposed to visible light. A special enzyme designated PRE, induced by visible light, splits or unlinks the dimers formed because of exposure to UV light and restores the DNA to its original state.

Some bacteria have enzymes, called *endonucleases* and *exonucleases*, that excise or cut out a damaged segment of DNA. Then the other enzymes, poly-



merases and ligases, repair the resulting break by filling in the gap and joining the fragments together. This mechanism is illustrated in Fig. 12-9 and is called excision repair.

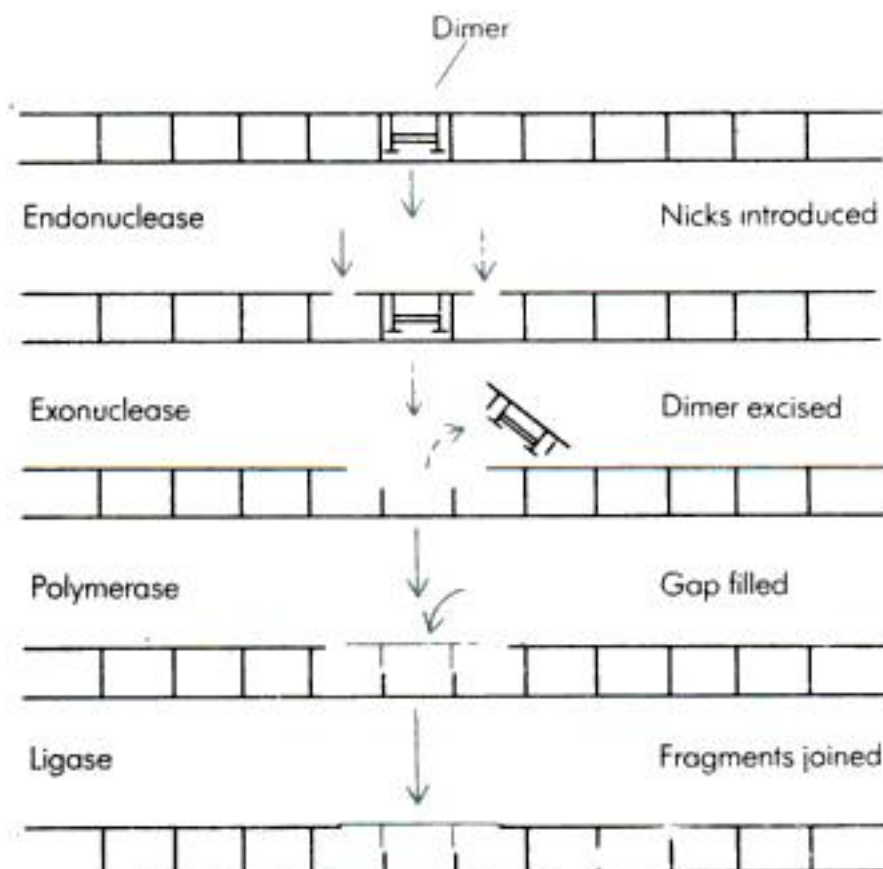
The process by which *E. coli* repairs large amounts of DNA damage is called **inducible or SOS repair**. This process is not a single discrete mechanism but includes diverse responses such as the ability to repair pyrimidine dimers, to induce various prophages, to shut off respiration, and to delay septum formation during cell division. But all the responses are coordinately regulated. The process is a very efficient one; however, it tends to insert mismatched bases and thus is error-prone and introduces additional mutation.

## Mutation Rate

The rate of mutation is the probability that a gene will mutate at any particular cell division. Thus the mutation rate is generally defined as the average number of mutations per cell per division. It is expressed as a negative exponent per cell division. For example, if there is one chance in a million that a gene will mutate when the cell divides, the mutation rate for any single gene equals  $10^{-6}$  per cell division. Generally, the mutation rate for any single gene ranges between  $10^{-3}$  and  $10^{-9}$  per cell division. Thus a mutation rate for *E. coli* may be given as  $5.8 \times 10^{-8}$  mutations per bacterium per cell division.

The mutation rate has some practical implications. Since genes mutate at random and independently of each other, the chance of two mutations in the same cell is the product of the single mutation rates for each. So, for example, if the mutation rate to penicillin resistance is  $10^{-8}$  per cell division and that to streptomycin resistance is  $10^{-6}$  per cell division, the probability that both mutations will occur in the same cell is  $10^{-8} \times 10^{-6}$ , or  $10^{-14}$ . This mutation rate is very low. For this reason, it is a common practice to give two antibiotics simultaneously in the treatment of some diseases. For example, a combination of penicillin G and streptomycin has been of proven value in treating streptococcal infections. A cell which has become resistant to one antibiotic is still likely to be inhibited or killed by the other.

**Figure 12-9.** Excision repair of UV-light-damaged DNA containing a thymine-thymine dimer generated by covalent links between adjacent bases.

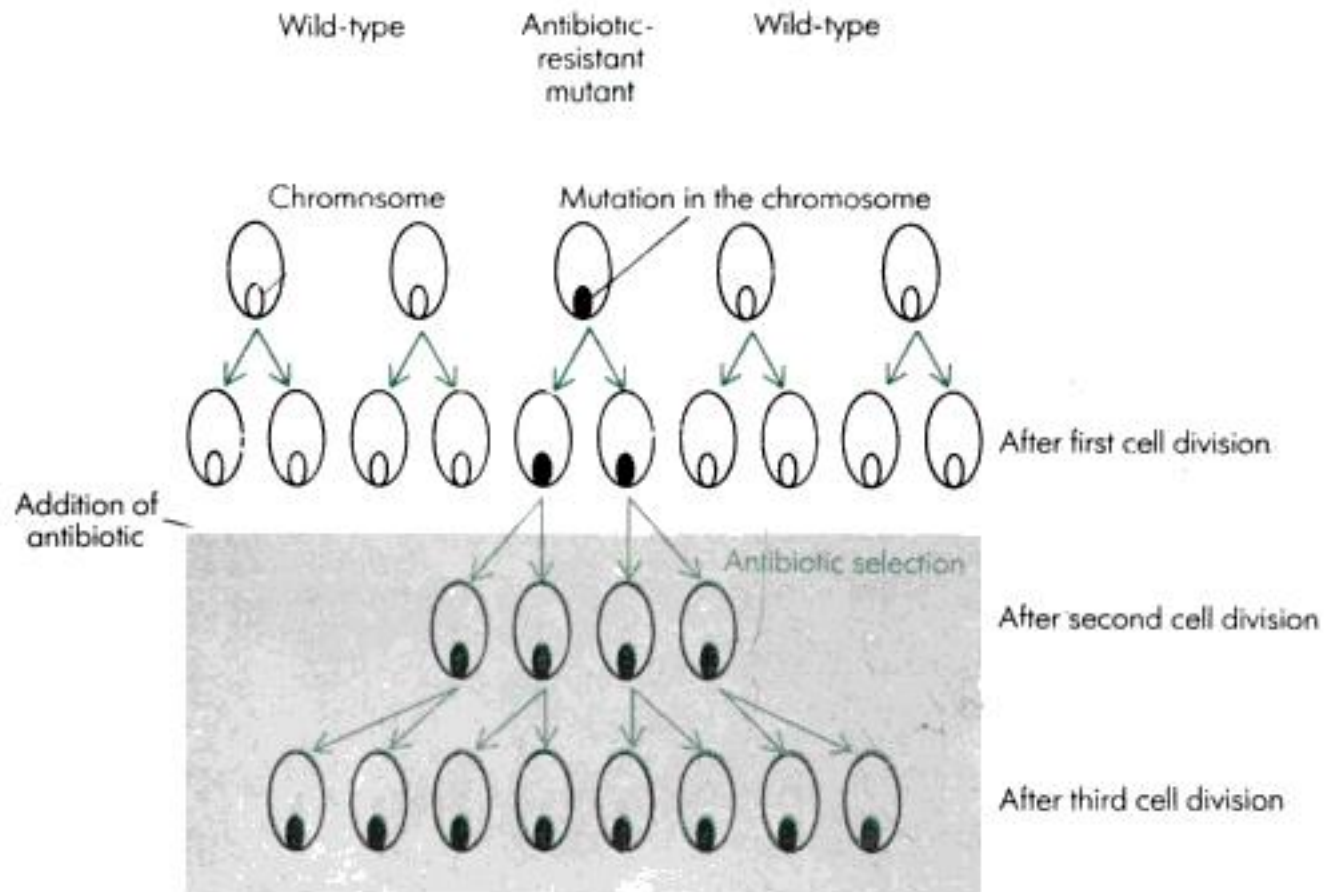




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**Figure 12-11.** Antibiotics originally effective for control of a bacterial infection become less effective or ineffective as antibiotic-resistant mutants appear. Note how the use of antibiotics (color screen) actually selects for bacteria that have become antibiotic-resistant due to chromosomal mutation. Bacteria sensitive to the antibiotic are killed or prevented from reproducing. (Courtesy of SANDORAMA 1978, Sandoz Ltd., Basel, Switzerland, and G. Lebek University of Berne.)



- 3 The maintenance of pure cultures of typical microorganism species requires that occurrence of mutation be prevented; otherwise, the culture will no longer be typical.
- 4 Microbial mutants have been extensively used in the investigation of various biochemical processes, particularly biosynthetic reactions. For example, mutants with blocks or impairment at different enzymatic steps have been used to unravel metabolic sequences.

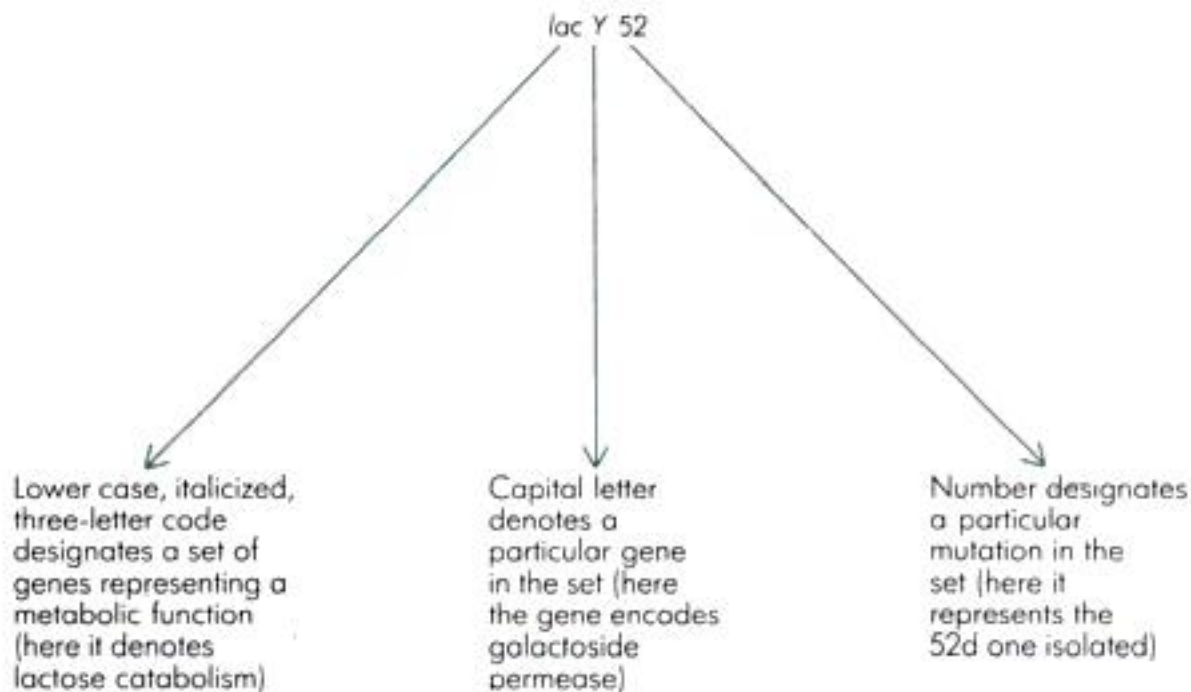
Many mutants, perhaps a majority, are able to revert to the wild-type condition by **reverse mutation**. This is a return to the original phenotype by the mutant cells. However, this may not necessarily be due to a precise reversal of the original mutation. Sometimes, the effect of the original mutation may be partially or entirely suppressed by a second mutation at a different site on the chromosome.

### Designation of Bacterial Mutants

The conventional designations used for bacterial mutants may be described briefly as follows. Each genotype is given a lowercase, italicized, three-letter code. For instance, a mutation which affects proline synthesis is designated *pro*. Since mutation in a number of different genes may exhibit identical phenotypes, discrete genetic loci can be differentiated by means of capital letters, for example, *proA*, *proB*, and *proC*. Numbers may be added sequentially to designate particular mutations; that is, as each new mutation is isolated, it is assigned a number that identifies it in bacterial pedigrees, for example, *proA52* is the 52d isolate of the *Escherichia coli* Genetic Stock Center at Yale University. A further example is given in Fig. 12-12. Table 12-1 shows some frequently encountered genotype abbreviations.

In referring to the phenotype of a bacterium, we use the same three-letter abbreviation, except that it is not italicized and its first letter is capitalized.



**Table 12-1.** Some Frequently Encountered Genotypes

Genotype	Mutation
<i>ala</i>	Alanine requirement
<i>azi</i>	Azide resistance
<i>div</i>	Cell division
<i>fla</i>	Flagella biosynthesis
<i>gal</i>	Galactose utilization
<i>lac</i>	Lactose utilization
<i>met</i>	Methionine requirement
<i>pur</i>	Purine biosynthesis
<i>str</i>	Streptomycin resistance
<i>thi</i>	Thiamine requirement
<i>ton</i>	Phage T1 resistance
<i>uvr</i>	Ultraviolet radiation sensitivity

**Figure 12-12.** Designation of bacterial mutations. The example used here is that of a mutation in *lac*-galactosidase synthesis.

Thus a mutant strain designated *pro* would be phenotypically  $Pro^-$  (which means inability to synthesize the amino acid proline). The superscript “+” would designate the wild type, for example,  $Pro^+$ . The “-” superscript represents the mutant.

## BACTERIAL RECOMBINATION

Genetic recombination is the formation of a new genotype by reassortment of genes following an exchange of genetic material between two different chromosomes which have similar genes at corresponding sites. These are called **homologous chromosomes** and are from different individuals. Progeny from recombination have combinations of genes different from those that are present in the parents. In bacteria, genetic recombination results from three types of gene transfer:

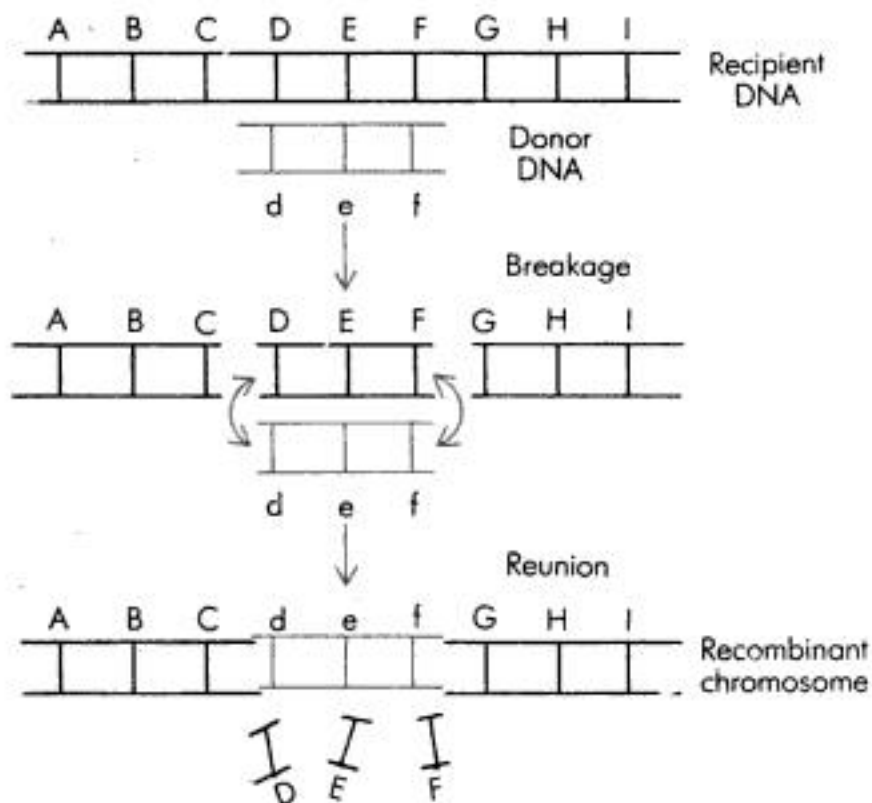
- 1 **Conjugation.** Transfer of genes between cells that are in physical contact with one another
- 2 **Transduction.** Transfer of genes from one cell to another by a bacteriophage
- 3 **Transformation.** Transfer of cell-free or “naked” DNA from one cell to another

These three types of gene transfer are shown in Fig. 12-13.

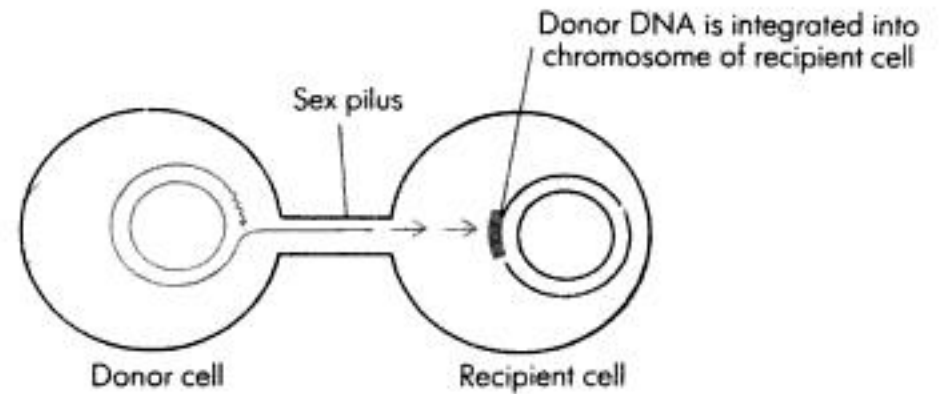
In bacterial recombination the cells do not fuse, and usually only a portion of the chromosome from the **donor cell** (male) is transferred to the **recipient cell** (female). The recipient cell thus becomes a **merozygote**, a zygote that is a partial diploid. Once merozygote transformation has occurred, recombination can take place.

The general mechanism for bacterial recombination is believed to take place as follows. Inside the recipient cell the donor DNA fragment is positioned alongside the recipient DNA in such a way that homologous genes are adjacent. Enzymes act on the recipient DNA, causing nicks and excision of a fragment.

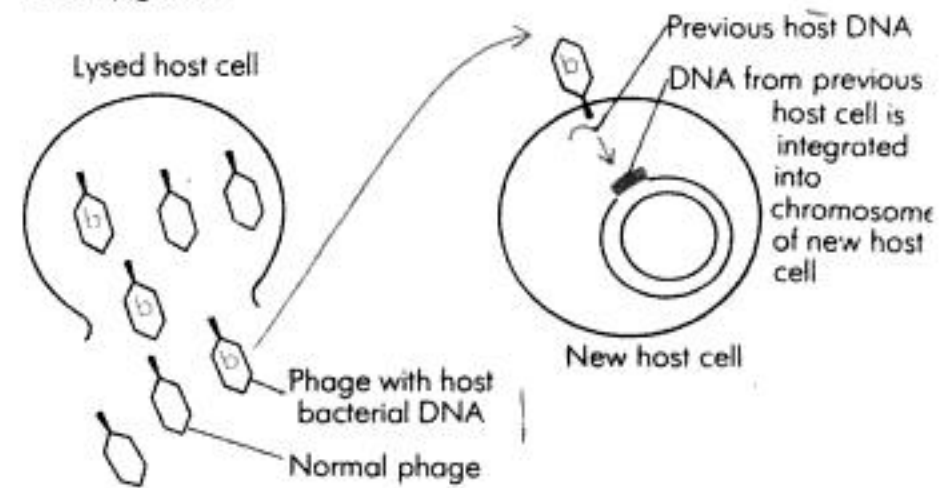
**Figure 12-13.** (Right) Three types of gene transfer from which genetic recombination results. (A) Conjugation, the transfer of genes between cells in physical contact with each other, perhaps by a sex pilus; (B) transduction, the transfer of genes between cells by a bacteriophage; (C) transformation, the transfer of cell-free or "naked" DNA from one cell to another.



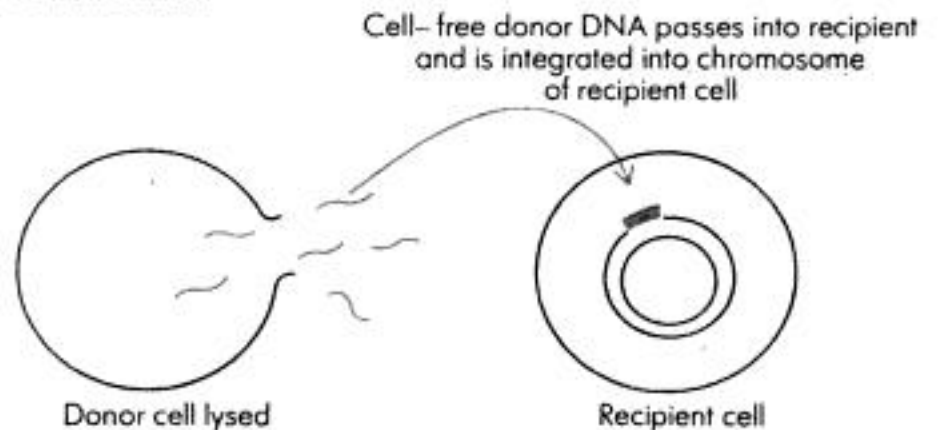
**Figure 12-14.** (Above) The breakage and reunion model of bacterial recombination. The donor DNA becomes integrated into the recipient DNA.



**A** Conjugation



**B** Transduction



**C** Transformation

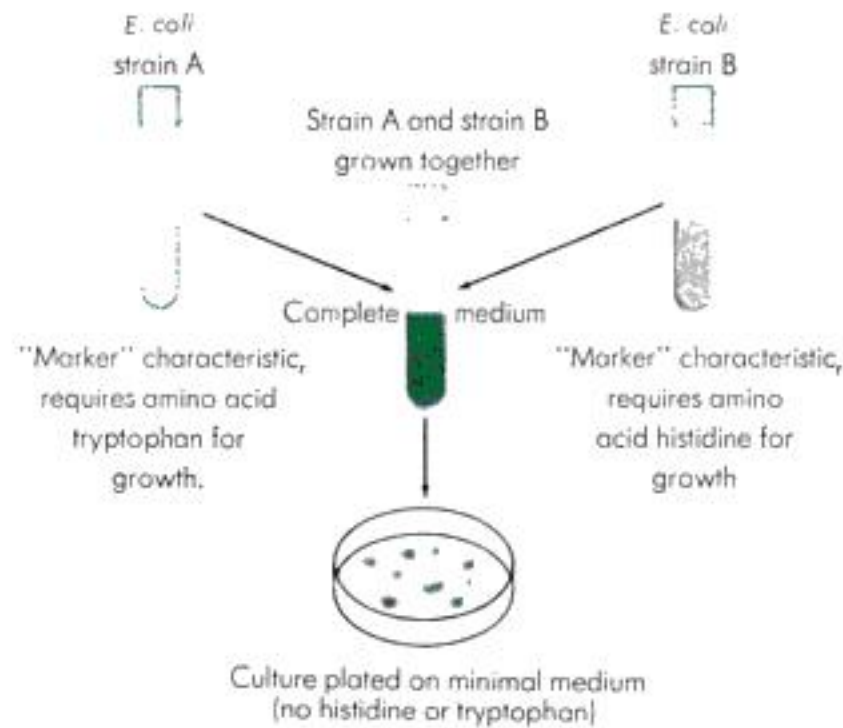
Then the donor DNA is integrated into the recipient chromosome in place of the excised DNA. The recipient cell then becomes the recombinant cell because its chromosome contains DNA of both the donor and the recipient cell. (The excised DNA pieces from the recipient chromosome are probably broken down by specific enzymes.) This general recombination mechanism is seen in Fig. 12-14.

## BACTERIAL CONJUGATION

Even though Luria and Delbrück had demonstrated in 1943 that bacteria have a stable hereditary system, it was impossible at that time to explore the system experimentally because of the lack of knowledge of any mating system in bacteria. The genetics of plants and animals depends upon the regular cycle of sexual reproduction in these organisms; once each generation, there is an opportunity for different mutants of a species to mate with each other and produce



**Figure 12-15.** Evidence for conjugation in bacteria. The two specific characteristics of the *E. coli* strains are (A) *trp*<sup>-</sup>, a tryptophan auxotroph, and (B) *his*<sup>-</sup>, a histidine auxotroph. The mixture is plated on a minimal medium; growth allows for the selection of prototrophic recombinants (organisms which can synthesize all their amino acid requirements).



new individuals with new combinations of mutations, i.e., to recombine with each other, or to produce **recombinants**. For example, a plant that produces smooth, yellow peas can be bred with one that produces wrinkled, green peas. Some of the next generation will be plants that produce the parental types—smooth and yellow or wrinkled and green. But other plants will produce the recombinant types smooth and green or wrinkled and yellow. Only by performing such crosses and observing the progeny can genetic work be done. The first demonstration of recombination in bacteria was achieved by Lederberg and Tatum in 1946 in a brilliant and remarkable experiment that opened the door to a whole new world of microbiology. Lederberg and Tatum knew that conjugation in bacteria must be quite rare, since no one had found it in spite of many attempts, and so they determined to select the few possible recombinants out of a large population. They combined two different auxotrophic strains of *E. coli* and gave them an opportunity to mate. Then they plated the combined cultures on a minimal medium, where only prototrophs could grow; when they found prototrophic colonies growing there, they knew that these must have been the result of a recombination between the auxotrophs.

Figure 12-15 shows the principle of their experiments in simplified form. When Lederberg and Tatum did their experiments, they used **polyauxotrophs** (mutants with more than one nutritional requirement) so that back mutation or spontaneous reversion to the wild type would not occur to confuse their results. For example, the probability of simultaneous reversion of three different mutations is of the order of  $(10^{-6})^3$ .

It had to be shown also that the prototrophs which arose could not have arisen by the phenomenon of transformation (discussed in the next section) since transformation had been discovered by Griffith in 1928. Cell-to-cell contact as a precondition was shown by using a U tube where the auxotrophic parental cells could be cultured together yet separated physically by a microporous fritted glass disk. This disk was permeable to macromolecules like DNA but not to cells. Culture fluid and soluble material could pass freely between the two parent cultures. Prototrophs or recombinants were never recovered from such



cultures. Result like these indicated that bacterial recombination by conjugation is indeed a true sexual process.

It is apparent that mating or conjugation in *E. coli* is radically different from sexual mating in higher organisms. It is not a reproductive process that occurs regularly at each generation. It does not involve meiosis since bacterial cells are haploid, nor does it involve the fusion of gametes. Instead, it involves the transfer of some DNA from one cell to another followed by separation of the mating pair of cells. While only very small fragments of the bacterial chromosome are transferred in transduction and transformation (discussed later), in conjugation it is possible for large segments of the chromosome, and in special cases the entire chromosome, to be transferred.

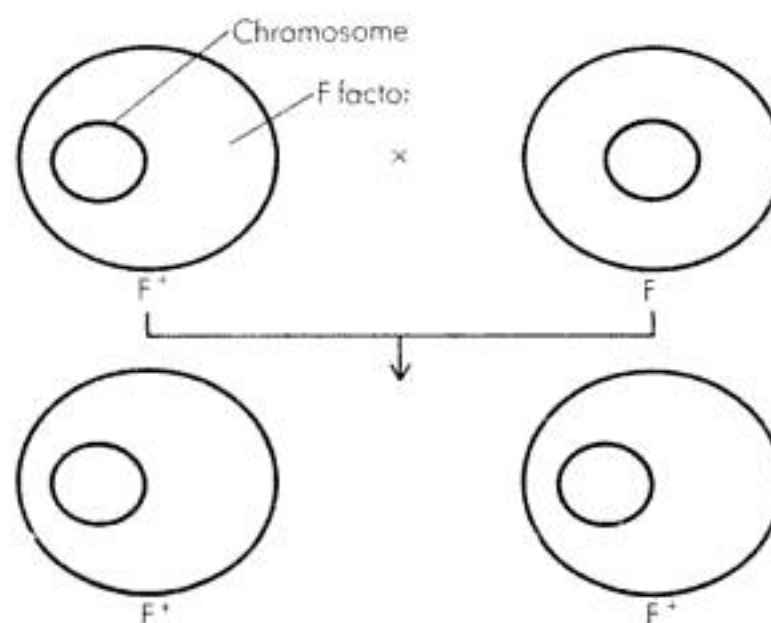
## Sex Factors

A clearer understanding of conjugation in bacteria came about with the discovery that there is sexual differentiation in *E. coli*; in other words, different mating types of the bacterium exist. Male cells contain a small circular piece of DNA, which is in the cytoplasm and not part of the chromosome, called the sex factor or F factor (fertility factor). These cells are referred to as  $F^+$  and are donors in mating. Female cells lack this factor and are labeled  $F^-$ . They are recipient cells.

Crosses between two  $F^-$  strains do not yield recombinants. However, in  $F^+ \times F^-$  crosses, the male replicates its sex factor, and one copy of it is almost always transferred to the female recipient. The  $F^-$  cell is converted to an  $F^+$  cell and is itself capable of serving as a donor (see Fig. 12-16). Therefore, as long as the cells grow, the conjugation process can continue in an infectious way with repeated transfer of the sex factor. The transfer of the F factor in an  $F^+ \times F^-$  cross occurs with a frequency that approaches 100 percent. But the formation of recombinants in an  $F^+ \times F^-$  cross occurs at a low frequency—about one recombinant per  $10^4$  to  $10^5$  cells. Thus we see that the transfer of the F factor is independent of the transfer of chromosomal genes.

Since the transfer of the F factor is independent, it follows that the F factor DNA replicates independently of the  $F^+$  donor cell's normal chromosome. The F factor DNA is only sufficient to specify about 40 genes which control sex-factor replication and synthesis of sex pili. One or more sex pili are produced by each  $F^+$  cell (Fig. 12-17). Sex pili seem to act to bind an  $F^-$  cell to an  $F^+$  cell and then to retract into the  $F^+$  cell, pulling the  $F^-$  cell into close contact. There is also some evidence that sex pili are tubules through which DNA passes

**Figure 12-16.** During mating of an  $F^+$  and  $F^-$  cell, the  $F^+$  cell replicates the sex or F factor and the copy is almost always transferred to the  $F^-$  cell. Thus an  $F^-$  cell usually becomes an  $F^+$  cell during mating.

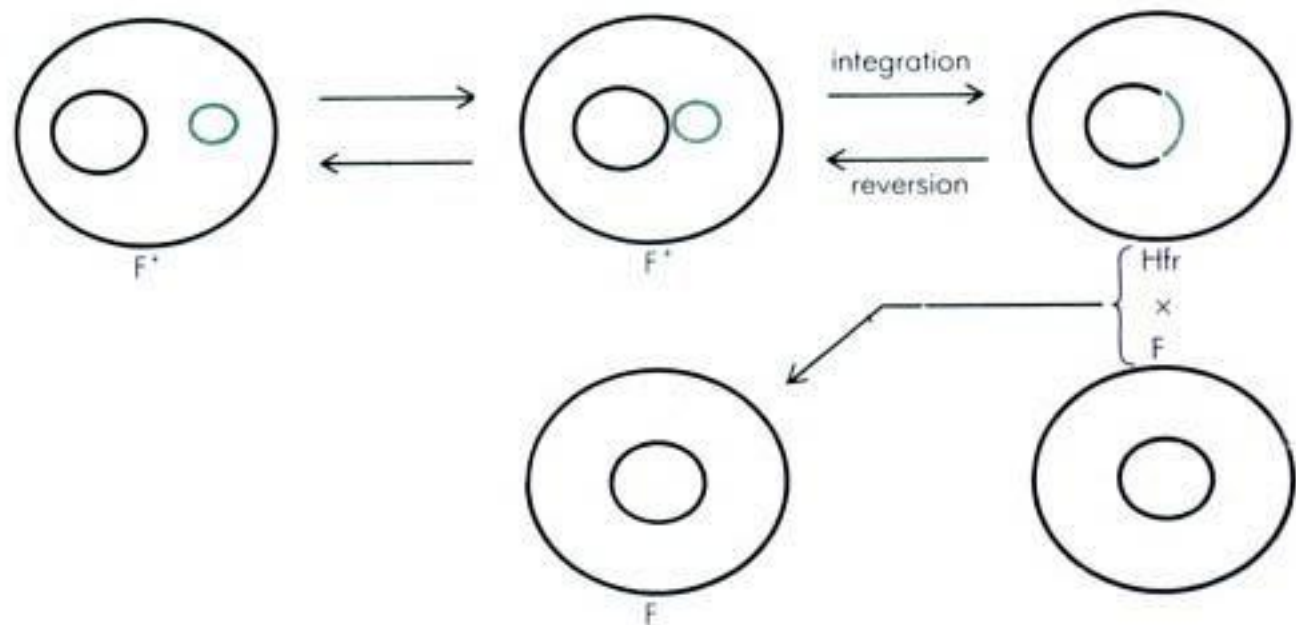




**Figure 12-17.** Sex pilus holding together a mating pair of *E. coli*. The male cell (on the right) also has another type of pili besides the sex pilus. Small RNA bacteriophages adsorbed to the sex pilus may be seen as dots. (X25,000.) (Courtesy of C. Brinton, Jr., University of Pittsburgh.)



**Figure 12-18.** An Hfr cell arises from an  $F^+$  cell in which the F factor becomes integrated into the bacterial chromosome. During mating of an Hfr and  $F^-$  cell, the  $F^-$  cell almost always remains  $F^-$ . This results because Hfr rarely transfers an entire F factor to the  $F^-$  cell. But the recombination frequency is high.



from an  $F^+$  to an  $F^-$  cell during conjugation, although the DNA may be passed from one cell to another at sites of contact between them.

### High-Frequency Recombination Strains

The study of conjugation in bacteria was made easier when new strains of cells were isolated from  $F^+$  cultures which underwent sexual recombination with  $F^-$  cells at a rate at least  $10^3$  times greater than  $F^+ \times F^-$  cells. These new donor strains were thus called **high-frequency recombination**, or **Hfr strains**. Hfr cells arise from  $F^+$  cells in which the F factor becomes integrated into the bacterial chromosome. They differ from  $F^+$  cells in that the F factor of the Hfr is rarely transferred during recombination. Thus in an  $Hfr \times F^-$  cross, the frequency of recombination is high and the transfer of F factor is low (Fig. 12-18); in an  $F^+ \times F^-$  cross, the frequency of recombination is low and the transfer of F factor is high.

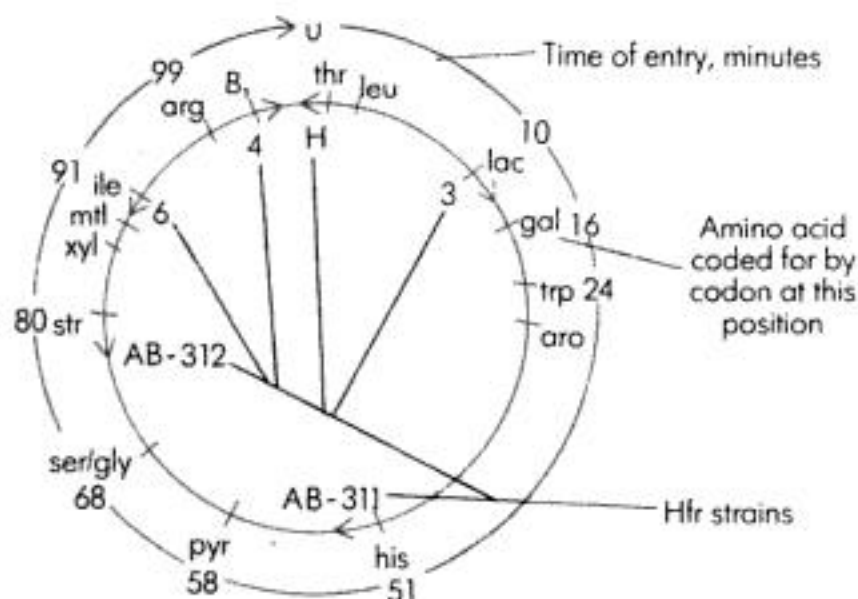
The order in which chromosomal material is transferred from an Hfr donor to an  $F^-$  recipient was determined by the interrupted mating experiments of



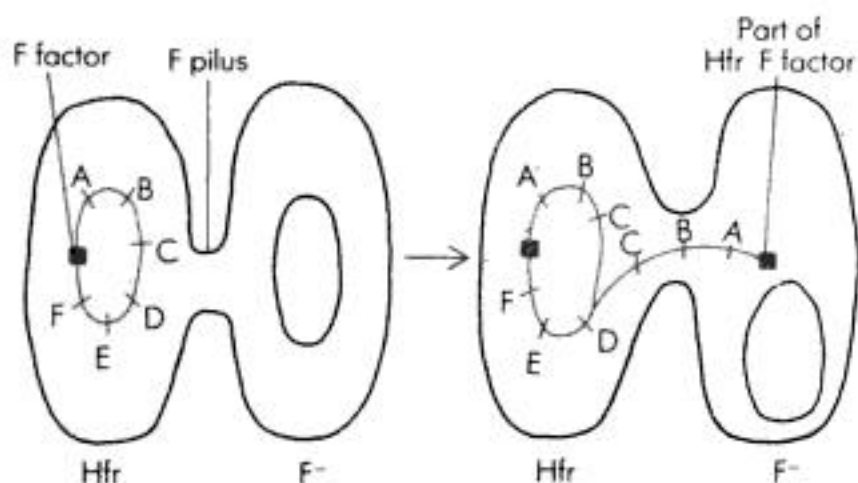
Elie Wollman and François Jacob. An Hfr strain was mixed with an  $F^-$  strain, and at various times the conjugation was interrupted by breaking the cells apart in a high-speed blender. The cells were then plated on various types of selective agar media in order to select for recombinant cells which had received donor genes before mating was interrupted.

Interrupted mating experiments reveal the order of genes on a chromosome by the time of entry and the frequency of recombination of each marker, which is a detectable mutation serving to identify the gene at the locus or site where it occurs. Each gene enters the  $F^-$  cell at a characteristic time, and a linkage map of the Hfr chromosome can be constructed using time of entry as a measure. This is the principal method of learning where the genes are on a bacterial chromosome (Fig. 12-19). This is all possible because the Hfr chromosome is transferred to the  $F^-$  cell in a linear fashion (Fig. 12-20) even though it is a circular chromosome. During transfer the Hfr chromosome begins replicating at the point of insertion of the F factor. Since the F factor can integrate in different positions of the Hfr bacterial chromosome, the first genes to enter an  $F^-$  cell will vary with different Hfr strains (Fig. 12-20). This means that the integrated F factor serves as the point of chromosomal opening, and part of it serves as the origin of transfer. The 5' end of the single DNA strand enters the  $F^-$  cell first. Data from an interrupted mating experiment is shown in Fig. 12-21.

It takes about 100 min to inject a copy of the whole Hfr *E. coli* genome (i.e., the chromosome and the integrated F factor). Since conjugation is usually in-

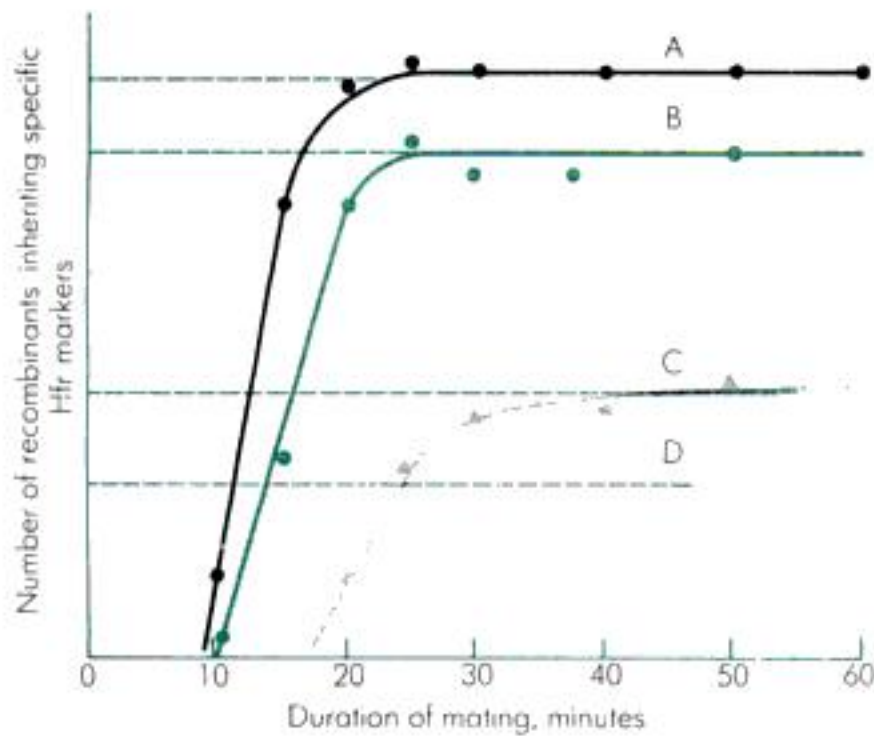


**Figure 12-19.** Simplified linkage map (color) of the circular chromosome of *E. coli* constructed from interrupted mating experiments using different Hfr strains. The arrows on the linkage map indicate the leading end and direction of entry of the chromosomes injected by each of the Hfr strains, the designations of which are shown inside the circle. This is determined by the position of the F factor in each of the Hfr strains. The numbers around the outside of the map show distances as a function of time, in minutes, based on time of entry of each codon in experiments. (Note that the map distances in minutes are drawn relative to the Hfr strain H so that the thr gene has been arbitrarily chosen as the origin.)



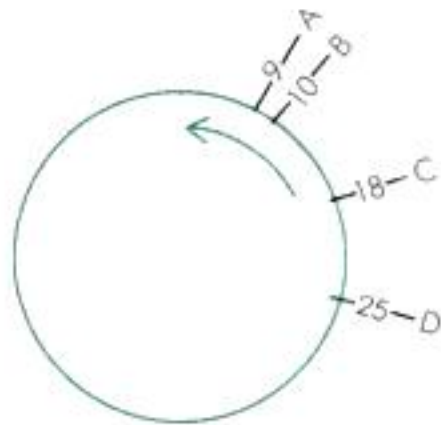
**Figure 12-20.** Mechanism for DNA transfer between Hfr and  $F^-$  cells. The Hfr chromosome begins replicating at the point of insertion of the F factor. Since the F factor can integrate in different positions of the Hfr bacterial chromosome, the first genes to enter an  $F^-$  cell will vary with different Hfr strains. As shown, the order of genes transferred is ABCDEF. In another Hfr strain, the F factor might be integrated between B and C. In this case the order of genes transferred would be CDEFAB.



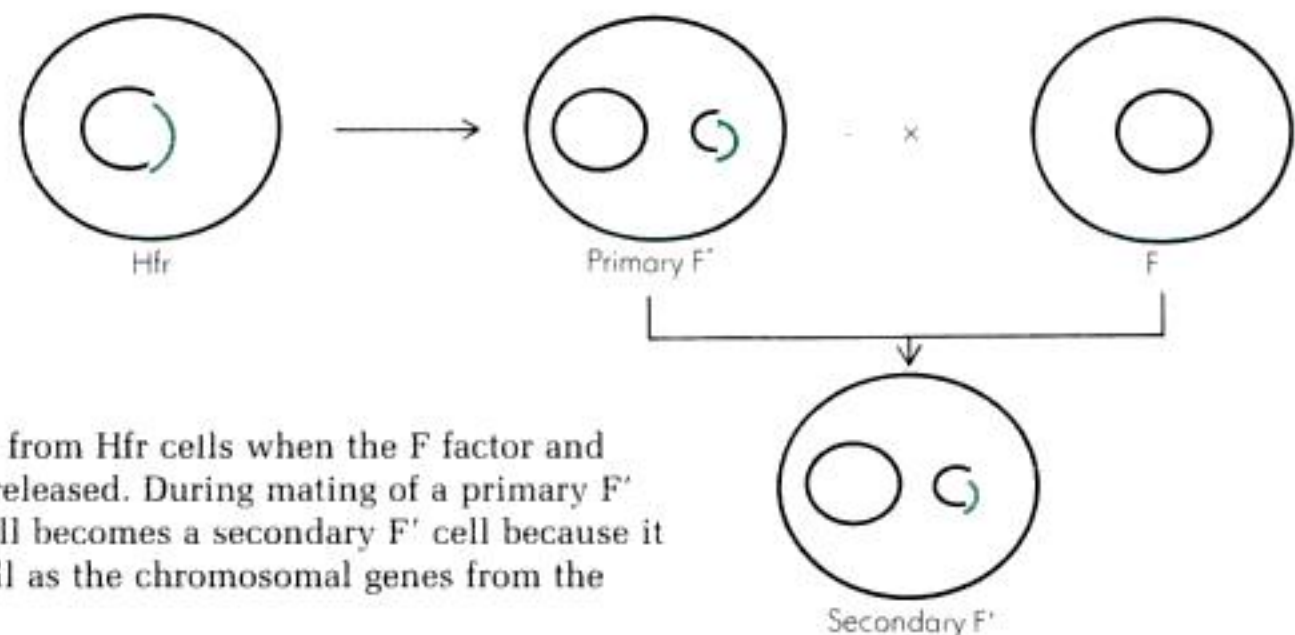


**Figure 12-21.** An interrupted mating experiment. (A) At intervals during the mating between Hfr and  $F^-$  cells, samples were removed from the culture and the conjugation interrupted. The cells were then plated on selective media that permitted growth of those  $F^-$  cells that inherited specific genetic markers received from the Hfr cells. In the graph, the number of recombinant colonies is plotted as a function of the interval of mating allowed before interruption. Extrapolation of each curve to zero recombinants gives the time of entry of the Hfr marker. As may be seen in the graph, the order of genes on the DNA is ABCD, and that A is very close to B. (B) Analysis of the data in (A) allows the plotting of a map as shown. Numbers denote minutes.

A



B

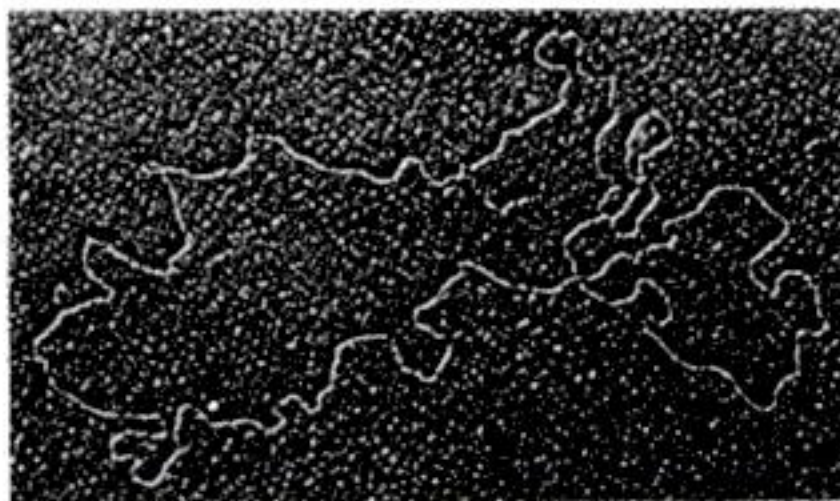


**Figure 12-22.** An  $F'$  cell arises from Hfr cells when the F factor and some chromosomal genes are released. During mating of a primary  $F'$  cell with an  $F^-$  cell, the  $F^-$  cell becomes a secondary  $F'$  cell because it now carries the F factor as well as the chromosomal genes from the primary  $F'$  cell.

interrupted by accident before this can occur, the distal Hfr genes are rarely transferred. Since *all* the Hfr chromosomal genes must be transferred before all the F factor genes are also transferred, most  $F^-$  recipients remain  $F^-$  after conjugation with Hfr cells.



**Figure 12-23.** Bacterial plasmid shown as a molecule of looped DNA. The drug-resistant plasmid shown is called R28K, carries ampicillin resistance, and has a length of 21  $\mu\text{m}$ . (Courtesy of Michiko Egel-Mitani.)



Hfr cells can revert to the  $F^+$  state. When this occurs, the sex factor is released from the chromosome and resumes its autonomous replication. Sometimes this detachment is not cleanly accomplished, so the  $F$  factor carries along with it some chromosomal genes. In this state it is termed an  $F'$  factor, and the cell in which this has occurred is called an  $F'$  cell (see Fig. 12-22). When such primary  $F'$  cells are mated with  $F^-$  recipients, the sex factor is transferred very efficiently together with the added bacterial genes. The recipient cell then becomes a secondary  $F'$  cell; it is a partial diploid for those genes it receives from the primary  $F'$  cell. This process whereby bacterial genes are transmitted from donor to recipient as part of the sex factor has been termed **sexduction** by Jacob and Wollman (Fig. 12-22).

### Extrachromosomal Genetic Elements (Plasmids)

In addition to the normal DNA chromosome, extrachromosomal genetic elements are often found in bacteria. These elements are called **plasmids** and are capable of autonomous replication in the cytoplasm of the bacterial cell (Figs. 11-12 and 12-23). Plasmids are circular pieces of DNA that are extra genes. Some plasmids are capable of either replicating autonomously or integrating into the bacterial DNA chromosome and are called **episomes**. Thus the  $F$  factor of *E. coli* was called an episome because it can alternately exist in the  $F^+$  or Hfr state.

Some bacteria have plasmids that are **bacteriocinogenic factors**. They determine the formation of **bacteriocins**, which are proteins that kill the same or other closely related species of bacteria. The bacteriocins of *E. coli* are called **colicins**; those of *Pseudomonas aeruginosa* are called **pyocins**, and so on. Bacteriocins have proved useful for distinguishing between certain strains of the same species of bacteria in medical bacteriological diagnosis. Bacteria possess other kinds of plasmids called **R plasmids** which confer resistance to a number of antibiotics. Some of the R plasmids can be transferred to other cells by conjugation, hence the term **infectious resistance**. Each form of resistance is due to a gene whose product is an enzyme that destroys a specific antibiotic.

## TRANSDUCTION

Most bacteriophages, the virulent phages, undergo a rapid lytic growth cycle in their host cells. They inject their nucleic acid, usually DNA, into the bacterium, where it replicates rapidly and also directs the synthesis of new phage proteins.



Within 10 to 20 min, depending on the phage, the new DNA combines with the new proteins to make whole phage particles, which are released by destruction of the cell wall and lysis of the cell. However, some bacterial viruses, the **temperate** phages, which ordinarily do not lyse the cell, carry DNA that can behave as a kind of episome in bacteria; like other episomes, such as the F factor, these viral genomes can become integrated into the bacterial genome; they are then known as **prophages**. Bacteria that carry prophages (**lysogenic** bacteria) can be induced with ultraviolet light and other agents to make the prophages start to replicate rapidly and go through a lytic growth cycle, resulting in lysis of the cell with release of new phage particles. (Bacteriophages are discussed in greater detail in Chap. 20.) Phage particles may become filled with cell chromosomal DNA or a mixture of chromosomal and phage DNA (rather than completely with phage DNA, as is normally the case). Such aberrant phages can attach to other bacteria and introduce bacterial, rather than just phage, DNA into them. By this means they transfer bacterial DNA from one cell to another. Thus we can define **bacterial transduction** as the transfer by a bacteriophage, serving as a vector, of a portion of DNA from one bacterium (a donor) to another (a recipient).

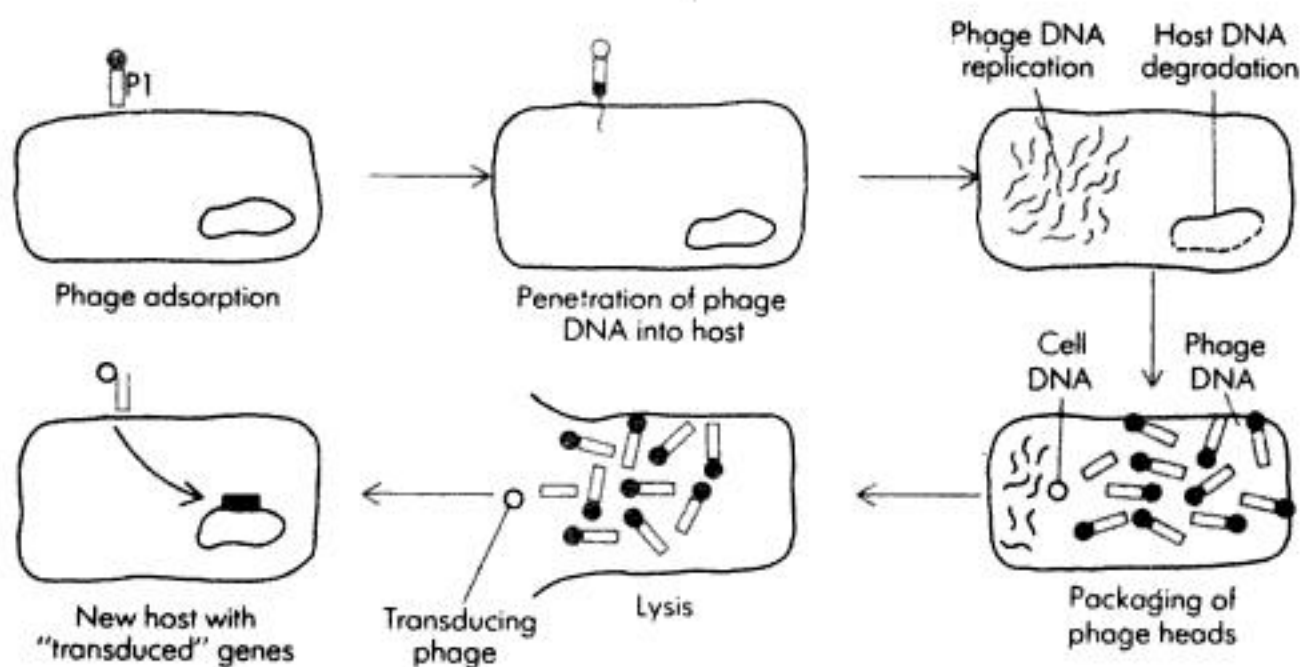
This phenomenon was discovered by Zinder and Lederberg in 1952 when they searched for sexual conjugation among *Salmonella* species. They mixed auxotrophic mutants together and isolated prototrophic recombinant colonies from selective nutritional media. When the U-tube experiment was carried out with a parental auxotrophic strain in each arm and separated by a microporous fritted glass filter, prototrophs appeared in one arm of the tube. Since the filter prevented cell-to-cell contact but allowed free passage of fluid between the cultures, it could be concluded that some phenomenon other than conjugation was involved. Furthermore, the phenomenon could not be prevented by DNAase activity, thus eliminating transformation as the process for changing the recipient auxotrophs to prototrophy. Further experiments implicated a bacteriophage as the vector or transducing agent in the following manner. The bacteriophage was released from a lysogenic (recipient) culture. The phage passed through the filter and infected the other strain (donor), lysing it. During replication in the donor strain, the phage adventitiously included parts of the bacterial chromosome with it. It then passed through the filter again, carrying part of the donor's genetic information and imparting it to the recipient strain.

### Generalized Transduction

If all fragments of bacterial DNA (i.e., from any region of the bacterial chromosome) have a chance to enter a transducing phage, the process is called **generalized transduction**. In this process, as the phage begins the lytic cycle, viral enzymes hydrolyze the bacterial chromosome into many small pieces of DNA. Any part of the bacterial chromosome may be incorporated into the phage head during phage assembly and is usually not associated with any viral DNA. For example, coliphage P1 can transduce a variety of genes in the bacterial chromosome. (This means that in a large population of phages there will be transducing phages carrying different fragments of the bacterial genome.) After infection a small proportion of the phages carry only bacterial DNA (see Fig. 12-24). The frequency of such defective phage particles is about  $10^{-5}$  to  $10^{-7}$  of the progeny phage produced. Since this DNA matches the DNA of the new



**Figure 12-24. Generalized transduction.** The phage P1 chromosome, after injection into the host cell, causes degradation of host chromosome into small fragments. During maturation of the virus particles, a few phage heads may envelop fragments of bacterial DNA instead of phage DNA. When this bacterial DNA is introduced into a new host cell, it can become integrated into the bacterial chromosome, thereby transferring several genes from one host cell to another.



bacterium infected, the recipient bacterium will not become lysogenic for P1 phage. Instead, the injected DNA will be integrated into the chromosome of the recipient cell. Defective P1 phages bearing *E. coli* DNA can be detected by the genetic markers present in that DNA. For instance, if a *thr*<sup>-</sup> cell is infected by a phage carrying a fragment of *E. coli* DNA with a *thr*<sup>+</sup> gene, this *thr*<sup>+</sup> gene may be integrated (recombined) into the bacterial chromosome to result in a prototrophic recombinant detectable by growth in a medium devoid of threonine.

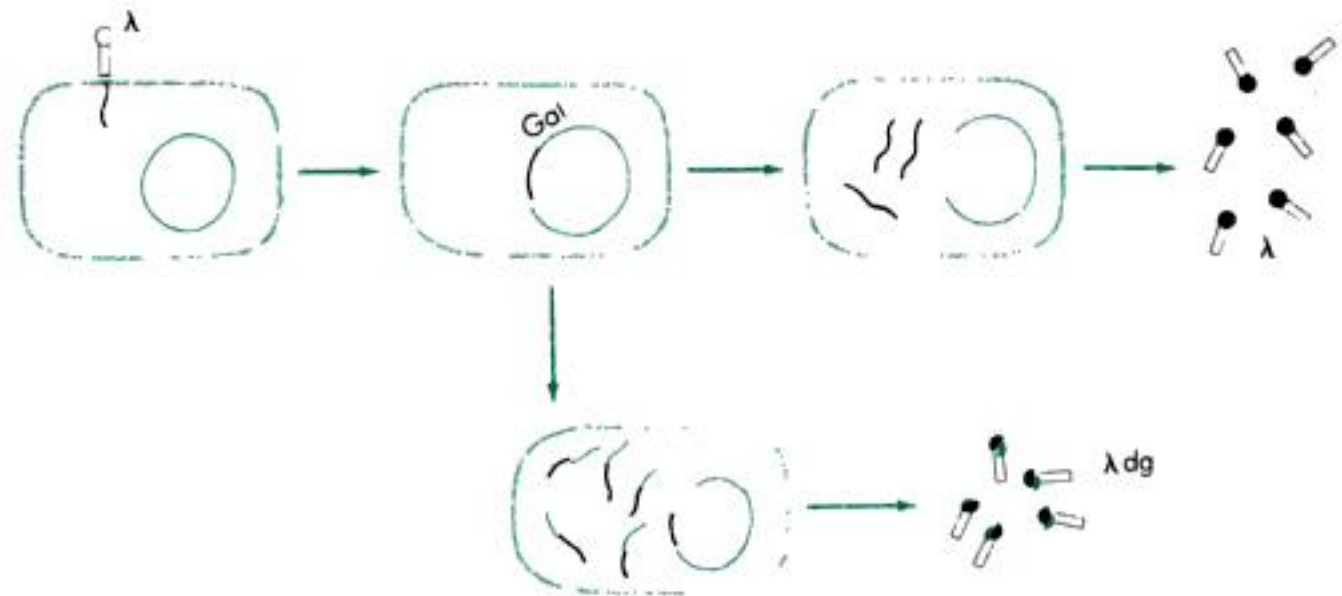
Generalized transduction, like bacterial conjugation and transformation, also provides a means for mapping bacterial genes, since the fragments transferred by a bacteriophage are often large enough to contain hundreds of genes. The mapping technique involves providing to the phage-infected bacteria a growth medium that selects for those recombinants that have inherited a given genetic marker from bacterial DNA carried by a transducing phage. Growth on other media can then be used to test how many of these recombinants have also inherited other donor markers. The closer two markers are together on the bacterial chromosome, the more likely they are to be inherited together by means of a single transducing phage. For example, when coliphage P1 is grown in a *thr*<sup>+</sup> *leu*<sup>+</sup> *azi*<sup>R</sup> host and then used to infect a *thr*<sup>-</sup> *leu*<sup>-</sup> *azi*<sup>-</sup> recipient bacterial cell, only 3 percent of the selected *Thr*<sup>+</sup> recombinants are also *Leu*<sup>+</sup>, and none are *Az*<sup>R</sup>, or azide-resistant. But, if *Leu*<sup>+</sup> recombinants are selected, about 50 percent of these are also *Az*<sup>R</sup>. This means that *leu*<sup>+</sup> is more closely linked to *azi*<sup>R</sup> than it is to *thr*<sup>+</sup> and the suggested order is therefore *thr*<sup>+</sup> *leu*<sup>+</sup> *azi*<sup>R</sup>. Thus the degree of linkage of genes can be measured by the frequencies of cotransduction of markers. The fact that only 3 percent of *thr*<sup>+</sup> transducing phages also carry *leu*<sup>+</sup> shows that these two genes are so far apart that they are rarely included at the same time in a DNA fragment that goes into the P1 head. (The P1 head carries a DNA molecule of slightly less than 10<sup>5</sup> nucleotide pairs.)

### Specialized Transduction

Bacterial genes can also be transduced by bacteriophage in another process called **specialized transduction**, in which certain temperate phage strains can transfer only a few restricted genes of the bacterial chromosome. More specifi-



**Figure 12-25.** Specialized transduction. When phage  $\lambda$  infects a cell, its DNA is inserted into the bacterial genome next to the genes for galactose metabolism (*gal* genes). Usually when such a cell is induced, the  $\lambda$  DNA comes out, replicates, and makes normal phage. However, occasionally the  $\lambda$  DNA is excised imperfectly, taking *gal* genes with it and leaving some of itself behind, leading to  $\lambda$ dg (defective, galactose transducing phage).



cally, the phages transduce only those bacterial genes adjacent to the prophage in the bacterial chromosome. Thus the process is also called **restricted transduction**. It occurs when a bacteriophage genome, after becoming integrated as prophage in the DNA of the host bacterium, again becomes free upon induction and takes with it into the phage head a small adjacent piece of the bacterial chromosome. In this way, when such a phage infects a cell, it carries with it the group of bacterial genes that has become part of it. Such genes can then recombine with the homologous DNA of the infected cell.

The best-studied specialized transducing phage is the phage lambda ( $\lambda$ ) of *E. coli*. The location of the  $\lambda$  prophage in the bacterial chromosome is almost always between the bacterial genes *gal* and *bio*. Whenever the phage genome comes out of, or is excised from, the bacterial chromosome, it sometimes takes with it *gal* or *bio* genes. When phages carrying *gal* or *bio* genes infect a new host, recombination with the *gal* or *bio* genes of the host can occur. This process is illustrated in Fig. 12-25. It should be noted that almost all phages that carry some bacterial genes because of "incorrect" excision are defective in certain viral functions because they are missing a piece of phage genetic information taken up by the bacterial genes. They cannot proceed through their entire replicative cycle, but the cell will yield phages if it is also infected with a complete phage that can code for the missing functions of the defective phages.

## BACTERIAL TRANSFORMATION

In 1928, an English health officer named Griffith injected mice with a mixture consisting of a few rough (noncapsulated and nonpathogenic) pneumococci and a large number of heat-killed smooth (capsulated and pathogenic) cells. (Living smooth pneumococci cause pneumonia in humans and other animals. "Rough" and "smooth" refer to the surface texture of the colonies of the respective cells.) The mice subsequently died of pneumonia, and live smooth cells were isolated from their blood. Apparently, some factor responsible for the pathogenicity of the smooth bacteria (even though they were dead) had been transferred to the living rough bacteria and had transformed them into pathogenic smooth ones. Griffith also showed that the transforming factor could be passed from the

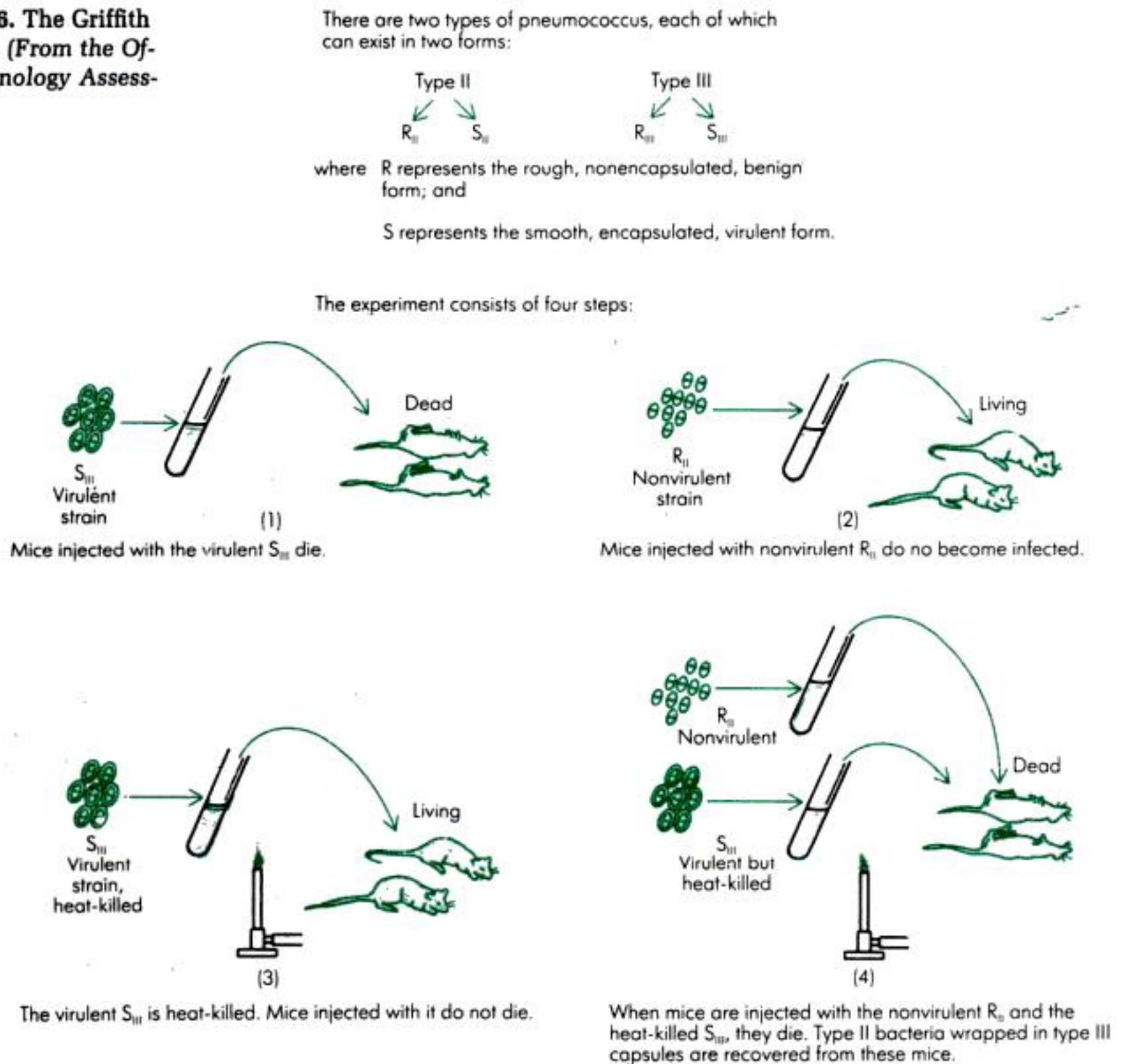


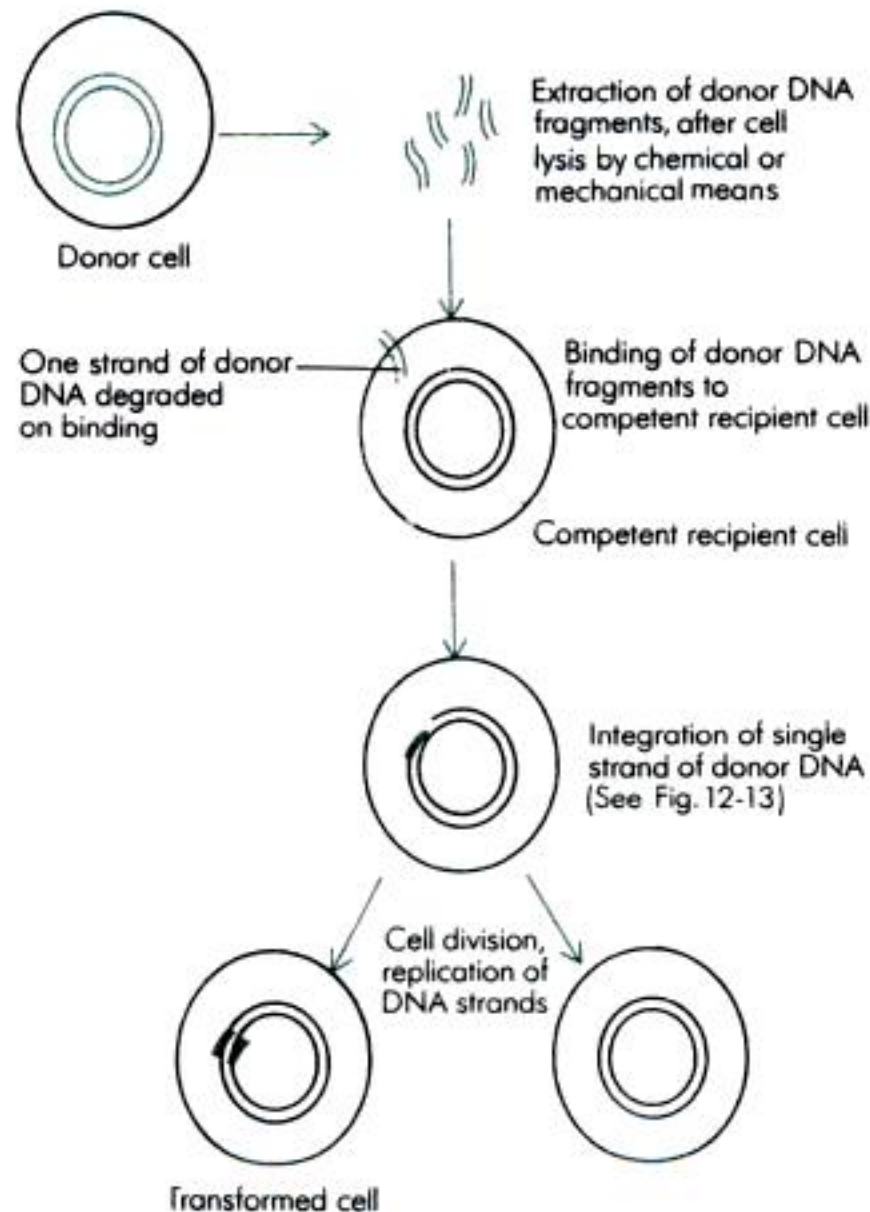
transformed cells to their progeny and thus had the characteristics of a gene. This experiment of Griffith is illustrated in Fig. 12-26.

This "transforming principle" was identified as DNA by Avery, MacLeod, and McCarty in 1944. They defined DNA as the chemical substance responsible for heredity.

Thus transformation is the process whereby cell-free, or "naked," DNA containing a limited amount of genetic information is transferred from one bacterial cell to another. The DNA is obtained from the donor cell by natural cell lysis or by chemical extraction. Once the DNA is taken up by the recipient cell,

**Figure 12-26.** The Griffith experiment. (From the Office of Technology Assessment.)





**Figure 12-27.** Principal steps in bacterial transformation.

recombination occurs. Bacteria that have inherited markers (specific characteristics) from the donor cells are said to be transformed. Thus certain bacteria, when grown in the presence of dead cells, culture filtrates, or cell extracts of a closely related strain, will acquire and subsequently transmit a characteristic(s) of the related strain.

The DNA is taken in through the cell wall and cell membrane of the recipient cell. The molecular size of the DNA affects transformation. Molecular weights of DNA in the range of 300,000 to 8 million daltons have been shown to result in successful transformation. The number of transformed cells increased linearly with increasing concentration of DNA. However, each transformation results from the transfer of a single DNA molecule of double-stranded DNA.

After DNA entry into a cell, one strand is immediately degraded by deoxyribonucleases, while the other strand undergoes base pairing with a homologous portion of the recipient cell chromosome; it then becomes integrated into the recipient DNA (see Fig. 12-27). Since complementary base pairing takes place between one strand of the donor DNA fragment and a specific region of the recipient chromosome, only closely related strains of bacteria can be transformed.

The principal steps of transformation are shown in Fig. 12-27. Bacterial species that have been transformed include, besides *Streptococcus pneumoniae*



(pneumococcus), those in the genera *Bacillus*, *Haemophilus*, *Neisseria*, and *Rhizobium*.

### Properties of Recipient Cells

Conditions suitable for uptake of donor DNA into recipient cells occur only during the late logarithmic phase of growth. During this period, the transformable bacteria are said to be **competent** to take up and incorporate donor DNA. Competent cultures probably produce an extracellular protein factor that apparently acts by binding or trapping donor DNA fragments at specific sites on the bacterial surface. The uptake process has been found to be an energy-requiring mechanism because it can be inhibited by agents that interfere with energy metabolism.

The significance of transformation as a natural mechanism of genetic change is questionable. It probably occurs following the lysis of a microbe and the release of its DNA into the environment. It is conceivable that transformation between bacterial strains of low **virulence** (disease-producing power of a microorganism) can give rise to transformed cells of high virulence. In any case, the phenomenon of transformation has proved to be extremely useful in genetic studies of bacteria in the laboratory, particularly in mapping the bacterial chromosome. This is because when DNA enters a recipient cell during transformation, the entering fragments of DNA are not unlike the DNA fragments transferred in a mating between Hfr and F<sup>-</sup> cells of *E. coli*. They will undergo **crossing-over** (exchange of portions of homologous chromosomes) with the homologous DNA segments of the recipient cells, and recombinants will be formed. As in conjugation, the frequency of transformation of two genes at the same time is an indication of the distance between these genes on the chromosome.

### THE REGULATION AND EXPRESSION OF GENE ACTIVITY

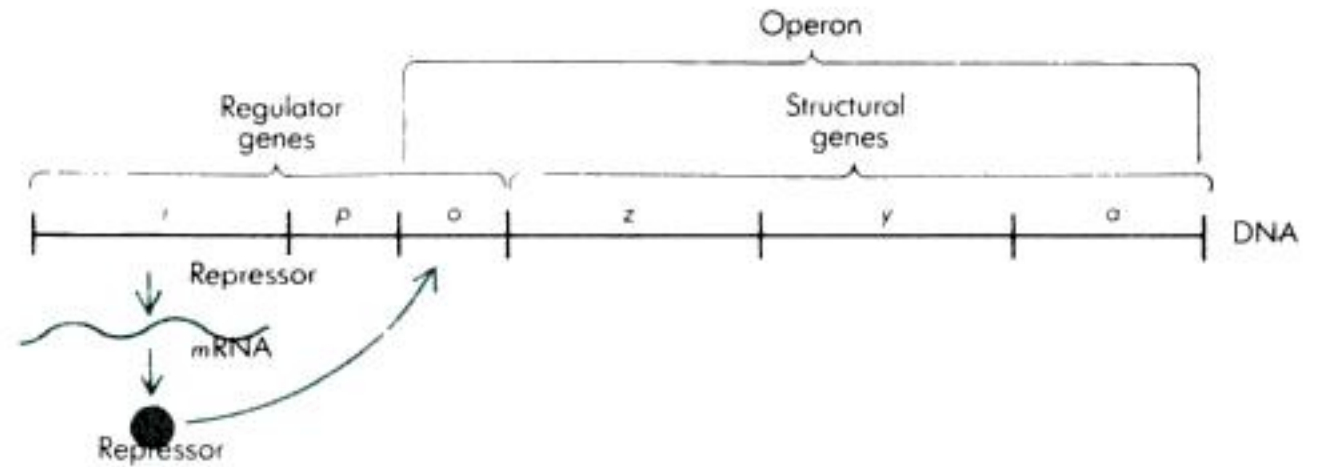
The regulation of gene activity is best controlled at the level of gene transcription. Many examples of such regulation have been discovered in *E. coli* and other bacteria.

Recall that in the bacterial chromosome the genes controlling the enzymes of a metabolic pathway are adjacent to each other. Several adjacent genes code for a single, long mRNA molecule that directs the synthesis of several enzymes of a specific metabolic pathway. The consequence of such an arrangement is that the amount of synthesis of gene products is **coordinately** regulated. Therefore, if a cell is stimulated to synthesize a large amount of one of the enzymes of a group, it will also make large amounts of the other enzymes of the same group. This kind of regulation involves the induction and repression of enzyme synthesis at the gene level and was discussed in Chap. 9. Maintenance of induction requires the continued synthesis of mRNA to balance its degradation. Thus this mRNA instability, coupled with transcriptional control, assumes that only necessary proteins are synthesized by the cell.

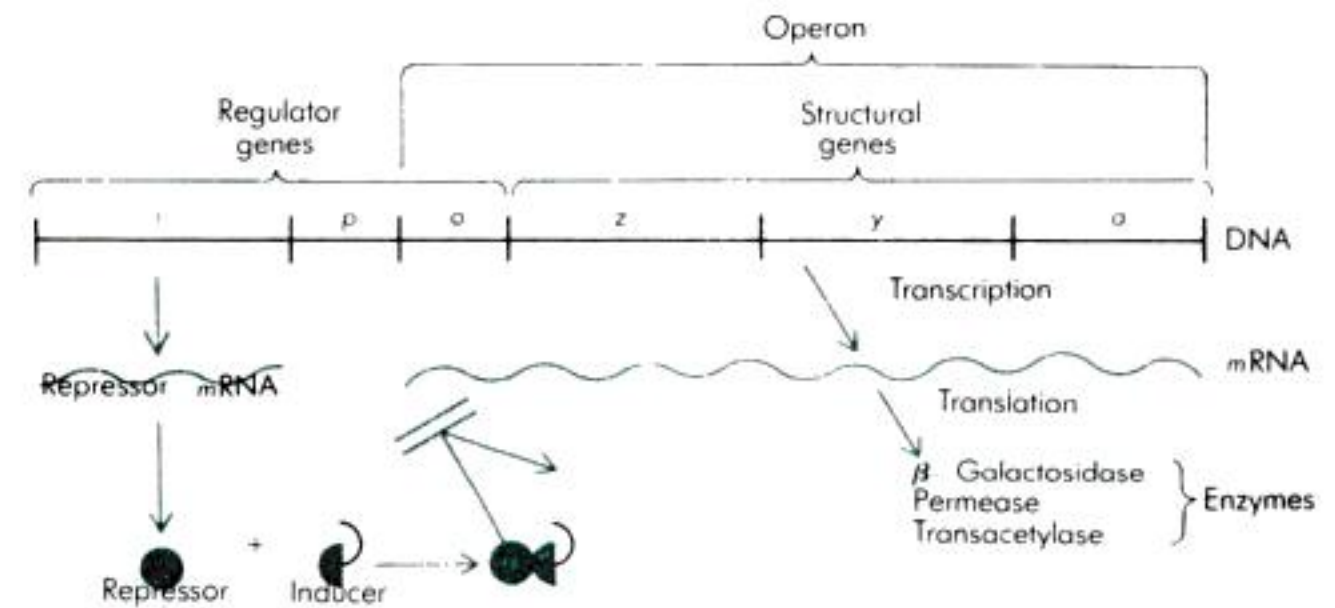
We can better understand the regulation of gene expression in procaryotes by discussing the lactose (*lac*) operon of *E. coli*. This operon is now by far the best understood part of any cellular genome. There are other bacterial operons, but they are less well understood and are different in detail from the *E. coli lac* operon.



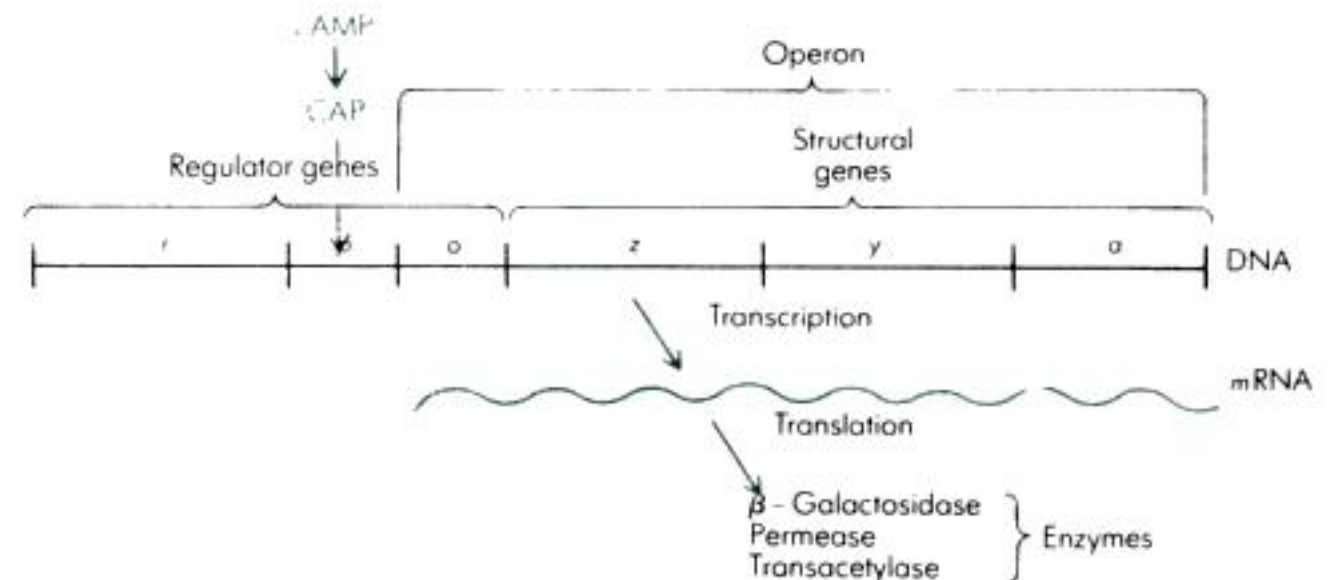
**Figure 12-28.** The Jacob-Monod model of gene control for the *lac* operon. (A) Repression of mRNA synthesis from *lac* operon. In the absence of inducer, the repressor (the product of the *i* gene) binds to the *o* gene to prevent transcription of the *z*, *y*, and *a* genes. (B) Induction of mRNA synthesis from *lac* operon. In the presence of inducer, the repressor binds to the inducer and can no longer combine with the *o* gene. The *lac* operon is no longer repressed, and transcription of the *z*, *y*, and *a* genes takes place. (C) Positive control of enzyme synthesis. Presence of cyclic AMP (cAMP) activates the catabolite gene activator protein (CAP), which in turn activates transcription of the *lac* operon.



**A** Repression of mRNA synthesis



**B** Induction of mRNA synthesis



**C** Positive control of enzyme synthesis



## The *lac* Operon

When inducers such as lactose or other  $\beta$ -galactosides are added to a culture of *E. coli*, there is a 1,000-fold increase in the rate of synthesis of the enzymes  $\beta$ -galactosidase (which hydrolyzes lactose to glucose and galactose),  $\beta$ -galactoside permease (which transports lactose into the cell), and thiogalactoside transacetylase (which plays no role in lactose utilization but may play a role in detoxifying certain thiogalactosides). The genes for these proteins are linked together on the *E. coli* chromosome. These are shown in Fig. 12-28 as *z*, *y*, and *a*, coding for  $\beta$ -galactosidase, galactoside permease, and transacetylase, respectively. In the absence of control, the rate of enzyme production would be constant and depend only on the structural genes (such as *z*, *y*, and *a*), amino acid levels, activating enzymes, and other substances. However, the control of the rate of enzyme synthesis is directed by the regulator genes designated *i*, *p*, and *o*, shown in Fig. 12-28, where *i* is the repressor gene, *p* the promoter gene, and *o* the operator gene. The *i* gene codes for a repressor protein which binds to the DNA of the operator *o* gene, thus preventing transcription, that is, the synthesis of mRNA (see Fig. 12-28). The promoter gene *p* is considered to be the site on the DNA where the RNA polymerase enzyme, catalyzing the synthesis of mRNA, binds, and is thus the site where the specific *lac* mRNA (responsible for the biosynthesis of the specific enzymes of the operon) synthesis begins. Let us discuss the functioning of the Jacob-Monod model of gene control for the *lac* operon as it is now understood.

- 1 Genes function as templates or blueprints for the transcription of mRNA. Using the protein-synthesizing machinery of the cell (ribosomes), the mRNA directs the synthesis of polypeptides (long chains of amino acids) in a process called translation.
- 2 The genes *z*, *y*, and *a* operate as a single unit of transcription, which is initiated at *p*.
- 3 Transcription of the operon is both negatively and positively controlled.

Negative control is mediated by the *lac* repressor which binds to the *o* gene and blocks transcription. Inducers, such as lactose, stimulate *lac* mRNA synthesis by binding to the repressor and reducing its affinity for the operator (see Fig. 12-28). Both repression and induction of enzyme synthesis are negative control systems because, in either case, the synthesis of enzyme can proceed only when the repressor is removed from its blocking site on the *o* gene.

Positive control of enzyme synthesis is said to occur when an association between a protein and a part of the regulatory region of an operon is essential for expression of related structural genes in the operon. Expression of the *lac* operon is inhibited when a more efficient source of energy, such as glucose, is present in the medium. The presence of glucose results in a decreased concentration of intracellular cyclic AMP (adenosine-3',5'-monophosphate). Cyclic AMP is necessary for efficient expression of the *lac* operon since it activates the catabolite gene activator protein (CAP), which in turn activates transcription of *lac* mRNA by RNA polymerase at the promoter site (see Fig. 12-28).

Thus both cyclic AMP and a specific inducer acting in concert are necessary for the synthesis of many inducible enzymes in *E. coli*. Little enzyme is made if either is absent.

The Jacob-Monod model of the *lac* operon has given biologists an insight into the molecular events of gene regulation. It shows up impressively the precision by which regulatory proteins modulate gene function: the repressors must recognize the specific nucleotide sequences of the operator gene on the one hand, and on the other hand they must recognize specific inducer molecules like lactose. An understanding of such regulatory mechanisms has been extended into the study of bacterial viruses.

Upon entering a bacterial host cell, the DNA genome of phage  $\lambda$  may either proceed to a developmental cycle leading to host-cell lysis or integrate into the chromosome of the host bacterium, making it lysogenic or temperate (see Fig. 12-25). Several phage genes are involved in deciding how fast a critical level of a specific repressor can be produced. When sufficient repressor is available, it blocks transcription of all the other phage genes by combining with two separate operators that control two important operons. In such a circumstance, no  $\lambda$  phage proteins are made, the host cell does not lyse, and the circular  $\lambda$  DNA is capable of being integrated into the host chromosome. If the repressors are destroyed or inactivated at any time during lysogeny of the bacterial cell, then the phage operons become derepressed and start functioning, the  $\lambda$  genome replicates, and the cell lyses. Thus it is seen that the repressor gene controls the fate of both the bacterial cell and the bacteriophage.

The mechanisms of the operon model of gene regulation are applicable only to procaryotic organisms and viruses. The same mechanisms have not been found to occur in eucaryotic cells in which the situation is more complex. For example, animal and human genes are full of "gibberish": segments of DNA that serve as coded genetic instructions are interrupted by other segments that have no function whatsoever. These extraneous pieces of DNA are called *introns* and often make up a larger portion of a gene than the actual code-bearing sequences (called *exons*). Thus the introns must all be spliced out of the genetic message before the cell can use it. It has been suggested that in eucaryotic cells regulation must involve controlling the functioning of the mRNA rather than its synthesis; that is, translation is controlled rather than transcription.

Furthermore, since cells with the same genome function differently in different organs in multicellular eucaryotic organisms, there must be some means for switching on and off whole sets of genes in particular cells. Since the chromosomes of eucaryotic cells do not exhibit any clustering of functionally related genes, any mechanism for controlling transcription must act, directly or indirectly, on many genes distant from each other. That is, there is coordinate control of many genes in different chromosomes.

In addition, it has been found that the mRNA that codes for a polypeptide does not always have the full sequence of nucleotides of the corresponding gene. Such mRNAs have been modified, after transcription, by splicing with specific enzymes.

Our knowledge of the mechanism of gene control in eucaryotic organisms is still fragmentary. But progress is slowly being made.

## GENETIC ENGINEERING

Genetic engineering refers to the development of organisms with genetic structure altered by biochemical manipulation. This kind of biochemical procedure is termed recombinant DNA technology and involves the use of plasmids as



well as certain bacteriophages. In a nutshell, this technology, developed in the early 1970s, consists of isolating, purifying, and identifying genetic material from one source; tailoring it for insertion into a new host; and isolating a colony of cells with the desired new genes. Since much of the results of genetic engineering have been utilized in biotechnology, this subject will be discussed in greater detail in Chap. 29.

## QUESTIONS

- 1 Explain how variability can arise in a bacterial culture.
- 2 Describe the advantages in using microorganisms for the study of genetics.
- 3 How do phenotypic changes differ from genotypic changes?
- 4 Compare the contribution of Max Delbrück and Salvador Luria with that of Esther and Joshua Lederberg in establishing that bacteria have a hereditary system.
- 5 What are point mutations, and what are their consequences?
- 6 What are frameshift mutations, and what causes them?
- 7 Explain the mechanism of photoreactivation.
- 8 What are transposons?
- 9 Why would it be desirable to give two antibiotics simultaneously in the treatment of some diseases?
- 10 Explain the notation used to describe bacterial mutants with respect to phenotypes and genotypes.
- 11 Describe some practical implications associated with the occurrence of microbial mutants.
- 12 What are the distinguishing characteristics of each of the three types of gene transfer?
- 13 How is sexual mating in bacteria like *Escherichia coli* different from that in higher organisms?
- 14 In what way did the discovery of sexual differentiation in *E. coli* contribute to an understanding of conjugation in bacteria?
- 15 Explain how interrupted mating experiments are used for determining the location of genes on a bacterial chromosome.
- 16 Describe the relationship between plasmids and episomes.
- 17 What are some conditions that facilitate the occurrence of transformation?
- 18 Differentiate between generalized transduction and specialized transduction.
- 19 Describe the events that take place during conjugation between an Hfr cell and an  $F^-$  cell of *E. coli*.
- 20 In what manner is the formation of a  $\lambda$ gal transducing phage similar to the formation of an  $F'$  factor?
- 21 Briefly explain how gene activity is controlled at the level of gene transcription.
- 22 Describe the molecular mechanism which governs the fate of the host cell upon infection with  $\lambda$  phage.

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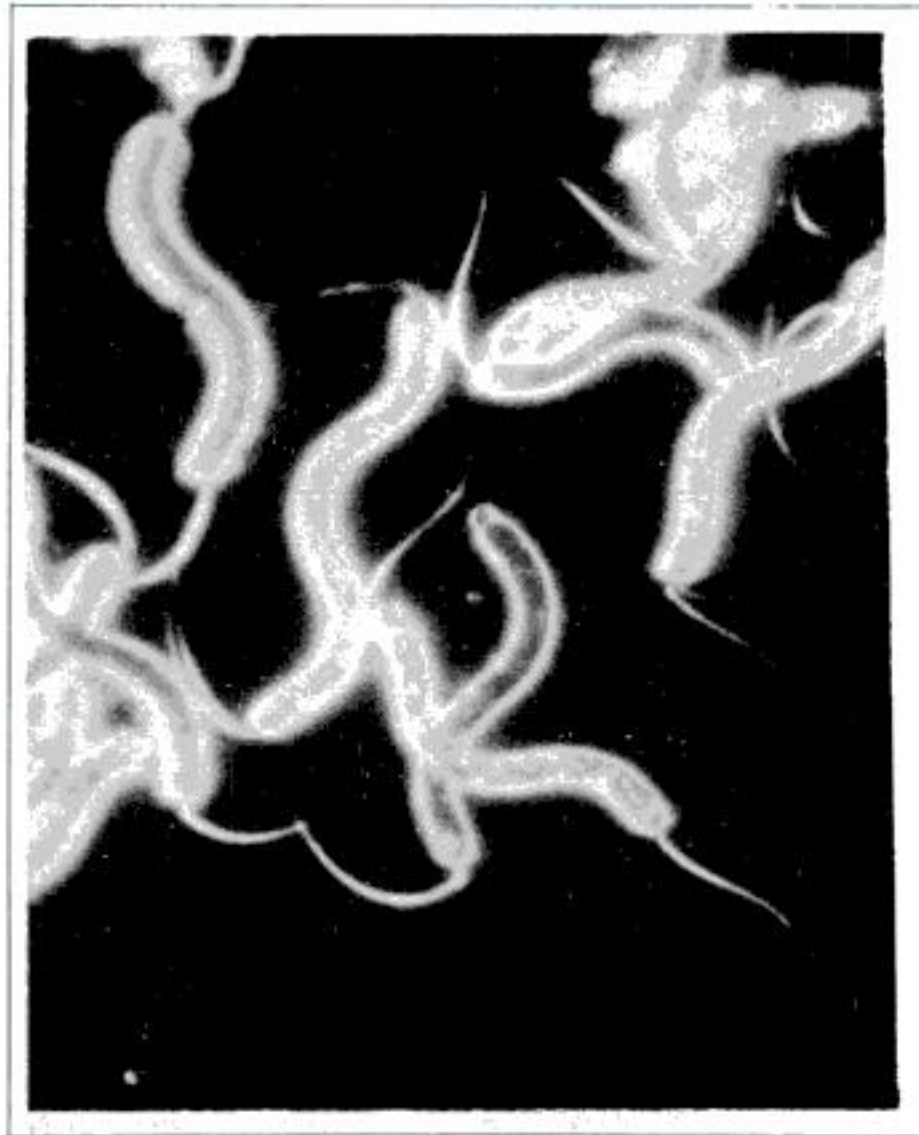
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# **PART FOUR**

## **THE WORLD OF BACTERIA**





### A mirror for the world of bacteria

Prior to 1923, many identification schemes for bacteria had been devised, but these schemes were usually fragmentary. There was need for a single, practical scheme that could cover all the described bacteria. From 1916 to 1918, Robert E. Buchanan was the first to prepare such a comprehensive scheme in a series of papers. In 1917, the Society for American Bacteriologists (now called the American Society for Microbiology) appointed a committee to coordinate all this information, and the final report from this committee in 1920, based largely on Buchanan's work, provided the beginning of a new outline for bacterial classification.

During this period, David H. Bergey began preparing a more complete review of the enormous literature of bacterial taxonomy. To aid the publication of this work, the Society of American Bacteriologists appointed an Editorial Board, chaired by Bergey. This resulted in the publication of the first edition of *Bergey's Manual of Determinative Bacteriology* in 1923.

Every taxonomic scheme for bacteria reflects the knowledge that exists at the time, and since knowledge constantly increases, bacterial taxonomy is subject to continual change. No one recognized this more clearly than Bergey and his colleagues. To cope with these changes, a second edition of the *Manual* was published in 1925, and a third edition in 1930. Five subsequent editions have appeared.

In 1934, the Society of American Bacteriologists transferred to Dr. Bergey all its rights, title, and interests in the *Manual* in order to allow Bergey to create an independent, nonprofit trust—The Bergey's Manual Trust. Throughout the years, this trust continues to

prepare and publish successive editions of the *Manual* and promotes research in the field of bacterial taxonomy.

Until 1974, the *Manual* was perceived largely as an "American classification" for bacteria (although it was becoming used in other countries as well), and it was prepared by a relatively small group of microbiologists. It owed its popularity to its breadth of coverage: it was the only book available that attempted to describe all the genera and species of bacteria. But knowledge about the properties of bacteria continued to accumulate at a nearly exponential rate. No longer were just a few people able to cope with the enormous number of bacterial taxa. Thus in 1974 the *Manual* began to become a truly international cooperative effort. Authorities from all over the world were invited to prepare the descriptions of the various genera and species, and the eighth edition of the *Manual* contained contributions from 135 authors.

In 1984, another major change occurred: the scope of the *Manual* was greatly broadened to bring together information dealing with the ecology, enrichment, isolation, preservation, and characteristics of bacteria, all of which concerned bacterial classification and identification. The new breadth of coverage was reflected by a new name—*Bergey's Manual of Systematic Bacteriology*. The new edition of this work is presently being prepared as four volumes, with contributions from hundreds of microbiologists.

As knowledge about bacteria continues to increase, so will *Bergey's Manual* continue to change with it and to act as a "mirror" for the world of bacteria.



## Chapter 13

# The World of Bacteria I: “Ordinary” Gram-Negative Bacteria

**OUTLINE** *Bergey's Manual of Systematic Bacteriology, Volume 1*

### The Spirochetes

**Aerobic/Microaerophilic, Motile, Helical/Vibrioid, Gram-Negative Bacteria**

**Nonmotile (or Rarely Motile), Gram-Negative Curved Bacteria**

### Aerobic Gram-Negative Rods and Cocci

The Family PSEUDOMONADACEAE • The Family AZOTOBACTERACEAE • The Family RHIZOBIACEAE • The Family METHYLOCOCCACEAE • The Family ACETOBACTERACEAE • The Family LEGIONELLACEAE • The Family NEISSERIACEAE • Other Genera of Aerobic Gram-Negative Rods and Cocci not Assigned to Any Family

### Facultatively Anaerobic Gram-Negative Rods

The Family ENTEROBACTERIACEAE • The Family VIBRIONACEAE • The Family PASTEURELLACEAE • Other Genera of Facultatively Anaerobic Gram-Negative Rods not Assigned to Any Family

### Anaerobic Gram-Negative Straight, Curved, and Helical Rods

The Family BACTEROIDACEAE

### Dissimilatory Sulfate- or Sulfur-Reducing Bacteria

### Anaerobic Gram-Negative Cocci

### The Rickettsias and Chlamydias

The Order RICKETTSIALES • The Family RICKETTSIACEAE • The Family BARTONELLACEAE • The Family ANAPLASMATACEAE • The Order CHLAMYDIALES

### The Mycoplasmas

The Family MYCOPLASMATACEAE • The Family ACHOLEPLASMATACEAE • The Family SPIROPLASMATACEAE

### Endosymbionts

The most widely used reference for bacterial classification is *Bergey's Manual of Systematic Bacteriology*, now published in four volumes. Volume 1 includes mainly the familiar or “ordinary” Gram-negative chemoheterotrophic eubac-

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The spirochetes comprise the order *SPIROCHAETALES*. This order is divided into two families, which are distinguished as follows:

THE FAMILY *SPIROCHAETACEAE*

- 1 They are stringent anaerobes, facultative anaerobes, or microaerophiles.
- 2 Carbohydrates or amino acids are used as carbon and energy sources.

THE FAMILY *LEPTOSPIRACEAE*

- 1 They are aerobes.
- 2 Long-chain fatty acids are used as the carbon and energy source.

Characteristics of the genera of these two families are indicated in Table 13-2.

**AEROBIC/MICROAERO-  
PHILIC, MOTILE, HELI-  
CAL/VIBRIOID, GRAM-  
NEGATIVE BACTERIA**

These bacteria have the following characteristics:

- 1 The cells are rigid (unlike spirochetes) and range from vibrioid (having less than one turn or twist) to helical (having one to many turns or twists).
- 2 They swim by means of polar flagella.

**Table 13-2.** Characteristics of the Genera of Spirochetes

Family and Genus	Habitat	Oxygen Relationships	Major Characteristics
<b><i>SPIROCHAETACEAE</i></b>			
<i>Spirochaeta</i>	Harmless inhabitants of water, mud, and sediments of marine and freshwater environments	Anaerobic and facultatively anaerobic	Use carbohydrates but not amino acids as carbon and energy sources
<i>Cristispira</i>	Harmless parasites of freshwater and marine molluscs	Unknown	Have never been isolated; have unusually large number of periplasmic flagella (>100)
<i>Treponema</i>	Mouth, intestinal tract, and genital areas of humans and animals; some are pathogenic	Anaerobic and microaerophilic	Use carbohydrate and amino acids; some have been cultivated in vitro (on nonliving media) and are stringent anaerobes; these are mainly harmless parasites, but one species, <i>T. hyodysenteriae</i> , causes hog dysentery.  Some species have not been cultivated in vitro, e.g., <i>T. pallidum</i> subsp. <i>pallidum</i> , which causes syphilis in humans and is microaerophilic
<i>Borrelia</i>	Parasites of wild rodents and small mammals, and also of the arthropods associated with these animals	Microaerophilic	Pathogenic, causing louseborne or tickborne relapsing fever in humans
<b><i>LEPTOSPIRACEAE</i></b>			
<i>Leptospira</i>	Some ( <i>L. biflexa</i> ) are harmless inhabitants of freshwater environments; others ( <i>L. interrogans</i> ) are parasites of wild and domestic animals	Aerobic	<i>L. interrogans</i> is pathogenic and causes leptospirosis in animals and humans



- 3 They are aerobic or microaerophilic.
- 4 They attack few or no carbohydrates.
- 5 The organisms usually give a positive reaction by the oxidase test (a laboratory test based on the presence of cytochrome c).

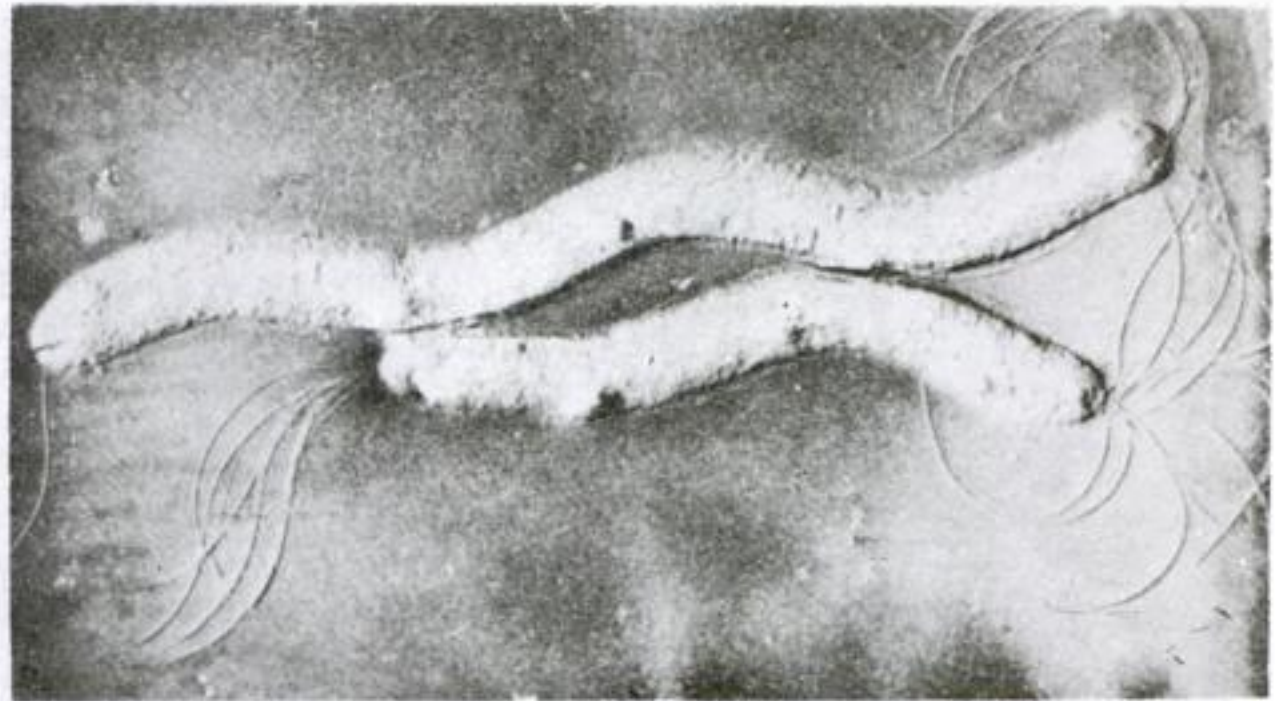
Most of the organisms are harmless saprophytes and occur in freshwater or marine environments, but a few are parasitic and can be pathogenic for humans and animals or for other bacteria. Some examples of genera in this section of Bergey's Manual are as follows:

### *Aquaspirillum*

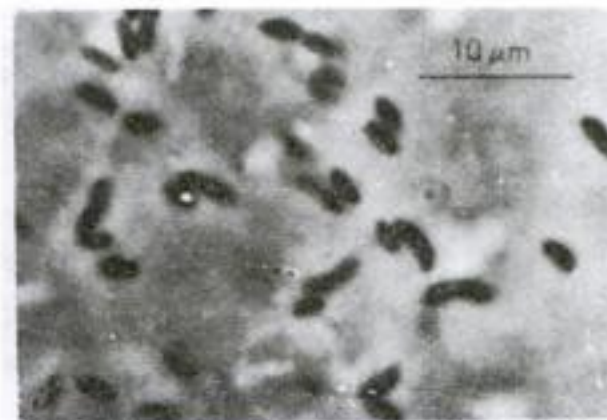
Aquaspirilla are helical or vibrioid organisms that typically possess bipolar tufts of flagella (see Fig. 13-2). These harmless saprophytes are aerobic to microaerophilic and occur in stagnant stream or pond water. No growth occurs in the presence of 3% NaCl or sea water.

### *Azospirillum*

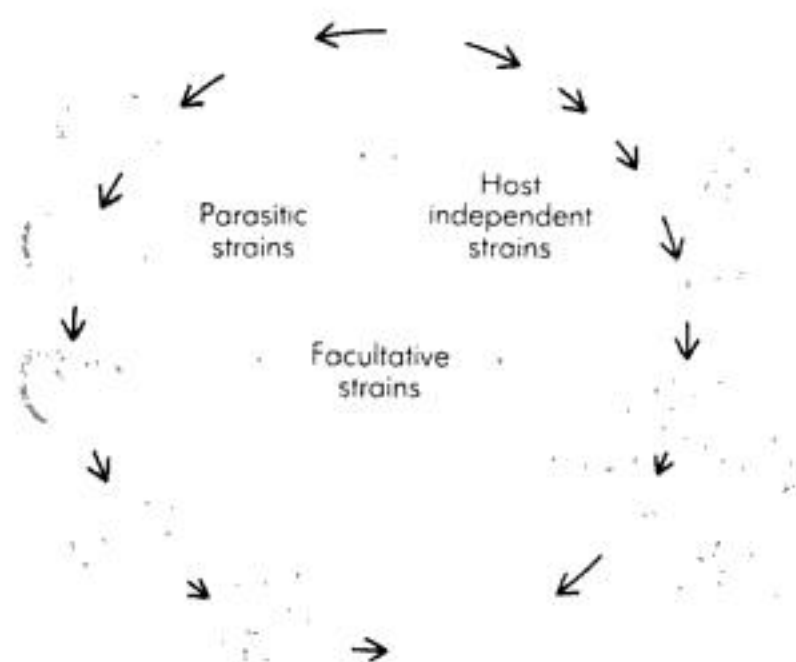
The cells are plump and vibrioid (see Fig. 13-3) with a single polar flagellum and, if grown on solid media, with numerous lateral flagella as well. Azospirilla occur within the roots of grasses, wheat, corn, and many other plants or as free-living soil organisms. They fix  $N_2$  within plant roots or in laboratory cultures. Under  $N_2$ -fixing conditions they are microaerophilic, but they are aerobic if supplied with a source of fixed nitrogen such as an ammonium salt. One species, *A. lipoferum*, can grow autotrophically with hydrogen gas as the energy source.



**Figure 13-2.** *Aquaspirillum bengal* (X9,800). (Courtesy of R. Kumar, A. K. Banerjee, J. H. Bowdre, L. J. McElroy, and N. R. Krieg, *Int J Syst Bacteriol*, 42:453, 1974.)



**Figure 13-3.** Plump vibrioid and straight cells of *Azospirillum brasilense* from a 48-h-old culture grown under nitrogen-fixing conditions. The cells are 1 μm in width. (Courtesy of N. R. Krieg, *Bacteriol Rev*, 40:55, 1976.)



**Figure 13-4.** Schematic representation of the life cycles of *Bdellovibrio*. All strains isolated from nature require host bacteria for growth (i.e., they are obligately parasitic). In the cycle depicted at the left, a bdellovibrio attaches to a host cell, penetrates the wall, grows within the periplasmic space into a long, coiled form that eventually fragments into new bdellovibrio progeny. Certain mutants can grow only on nonliving culture media (host-independent cycle, shown on the right). Rare strains are apparently facultative parasites and will grow in culture media or in host cells. (Courtesy of J. C. Burnham, T. Hashimoto, and S. F. Conti, *J Bacteriol* 101:997, 1970.)

### *Oceanospirillum*

The cells are helical, usually with bipolar tufts of flagella. *Oceanospirilla* are aerobic and are harmless saprophytes, occurring in coastal marine waters. Sea water is required for their growth.

### *Campylobacter*

These vibrioid cells have a single flagellum at one or both poles (see Fig. 36-4). *Campylobacters* are microaerophilic parasites, occurring in the reproductive organs, intestinal tract, and oral cavity of humans and other mammals. Some species are pathogenic; e.g., *C. jejuni*, which causes diarrhea in humans, or *C. fetus* subspecies *venerealis*, which causes abortion in cattle.

### *Bdellovibrio*

These aerobic, vibrioid cells possess a single polar flagellum. *Bdellovibrios* have the unique property of being parasitic on other Gram-negative bacteria. After attachment to a host bacterium, the bdellovibrio penetrates the outer membrane of the cell wall and grows within the periplasmic space. Eventually the host bacterium becomes an empty "ghost" cell. This life cycle is illustrated in Fig. 13-4. *Bdellovibrios* occur in soil, sewage, and in freshwater and marine environments. The genus *Vampirovibrio* has certain similarities to the genus *Bdellovibrio*, but the organisms attack eucaryotic algae, not bacteria.

### NONMOTILE (OR RARELY MOTILE), GRAM-NEGATIVE, CURVED BACTERIA

The characteristics of the bacteria in this section are as follows:

- 1 Rigid cells that are curved to various degrees, forming coils, helical spirals, and sometimes rings (i.e., cells that are curved around so that the ends overlap (Fig. 13-5))
- 2 Nonmotile

These harmless saprophytes occur mainly in soil, freshwater, and marine environments. One example is the family *Spirosomaceae*, which contains three genera whose cells, which are aerobic, form no intracellular gas vacuoles, are catalase- and oxidase-positive, and form colonies that are yellow (the genus



## AEROBIC, GRAM-NEGATIVE RODS AND COCCI

### The Family PSEUDOMONADACEAE

#### *Pseudomonas*

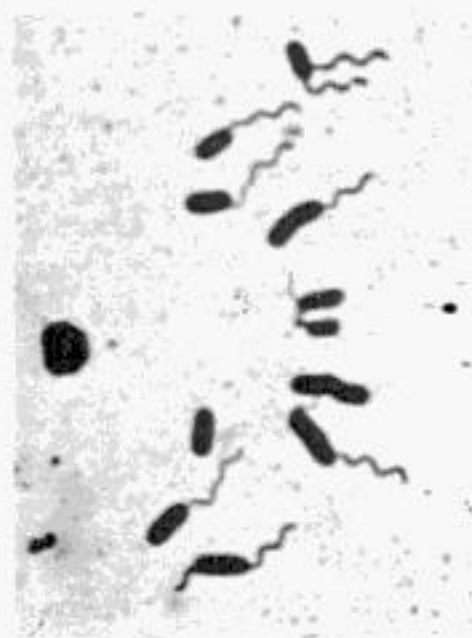


Figure 13-6. (Above) Flagella stain (X2,000) of cells of a *Pseudomonas* strain showing the characteristic polar flagella. (Courtesy of General Biological Supply House, Inc.)

*Spirosoma*) or pink (the genera *Ranella* and *Flectobacillus*). Another common genus, not included in the family Spirosomaceae, is *Microcycilus*, which forms prominent intracellular gas vacuoles and whose colonies have no pigment.

This section forms one of the largest and most diverse groups of bacteria. Two general features are as follows:

- 1 The cells are mainly straight or slightly curved (but not helical) rods, but some are cocci.
- 2 They have a strictly respiratory type of metabolism.

Several families and some additional genera that are not assigned to any family are represented.

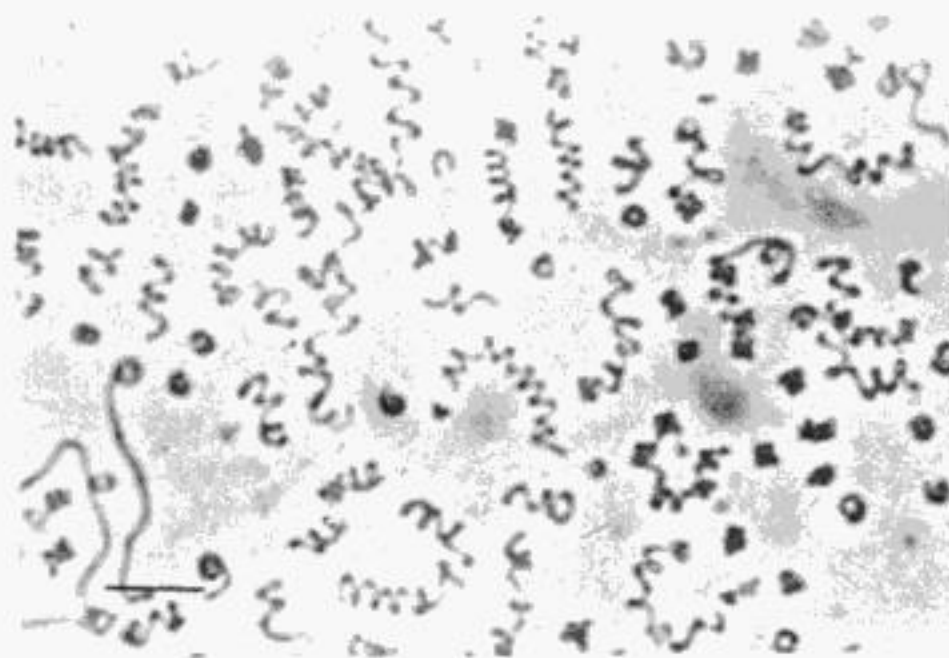
The features of this family include the following:

- 1 Straight or slightly curved rods
- 2 Motile by polar flagella (Fig. 13-6)
- 3 Catalase-positive and usually oxidase-positive

Some of the genera included in this family are described here.

These bacteria are widely distributed in soil and water. The genus contains five genetically distinct groups (each of which might be considered as a separate genus) as well as a large number of additional, less well studied organisms. All pseudomonads can grow aerobically, but some can also grow anaerobically by using nitrate as an electron acceptor. Several species are pathogenic for humans or animals; others are important plant pathogens. Some cause spoilage of meats and other foods. Species identification is based on many physiological and nutritional characteristics, such as the ability to use certain compounds as carbon sources for growth. Some species can use any of over 100 compounds as a carbon source. Sugar-containing media are acidified only weakly, and acid

Figure 13-5. (Below) Phase-contrast photomicrograph of *Spirosoma* cells showing rings and coils. The bar indicates 10  $\mu$ m. (Courtesy of J. M. Larkin, P. M. Williams, and R. Taylor. In *J Syst Bacteriol* 27:147, 1977.)



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### The Family *RHIZOBIACEAE*

#### *Rhizobium* and *Bradyrhizobium*

This family contains rod-shaped cells that incite hypertrophies on plants (root nodules, leaf nodules, or tumors). Three genera of this family are described below.

These bacteria fix  $N_2$  by means of a complex, highly evolved symbiosis with the roots of leguminous plants. The bacteria attach to the root hairs, penetrate the root, and induce proliferation of the root cells (see Figs. 25-11 and 25-12). Within the resulting root nodules the bacteria exist as highly pleomorphic  $N_2$ -fixing forms called bacteroids. Leghemoglobin occurs within the root nodules and serves to protect the nitrogenase enzyme complex from being destroyed by excess oxygen. The species and strains of rhizobia and bradyrhizobia exhibit a range of specificities for various legumes.

#### *Agrobacterium*

Agrobacteria do not fix  $N_2$ . The organisms are plant pathogens that incite tumors when they invade the crown, roots, and stems of a great variety of dicotyledonous and some gymnospermous plants (see Fig. 36-20). Tumor induction is associated with the presence in the bacteria of a particular plasmid (see Chap. 36 for further details).

### The Family *METHYLOCOCCACEAE*

This family consists of a diverse group of rods, vibrios, and cocci having in common the ability to use methane gas as a sole carbon and energy source under aerobic or microaerophilic conditions. These harmless organisms occur in soil, mud, or water adjacent to or overlying the anaerobic environments where methane is formed. Some members of the family fix nitrogen under microaerophilic conditions. Some form *Azotobacter*-like cysts. The genera presently included—*Methylococcus* and *Methylomonas*—are all obligate methane-oxidizers (i.e., carbon sources such as glucose cannot be used for growth); however, the definition of the family permits inclusion of facultative methane-oxidizers as well.

### The Family *ACETOBACTERACEAE*

This family contains ellipsoidal to rod-shaped cells that oxidize ethanol to acetic acid in neutral or acidic (pH 4.5) media. Two genera are included, *Acetobacter* and *Gluconobacter*, which are differentiated by certain biochemical characteristics and by the occurrence of peritrichous flagella (*Acetobacter*) or polar flagella (*Gluconobacter*).

Members of these two genera are saprophytes that occur in sugar- or alcohol-enriched, acidic environments such as flowers, fruits, beer, wine, cider, vinegar, souring fruit juices, bees, and honey. Some have industrial importance: acetobacters are used to make vinegar, and gluconobacters are involved in the manufacture of chemicals such as dihydroxyacetone, sorbose, and 5-ketogluconic acid (see Table 29-1). Some strains of *Acetobacter* have the highly unusual ability (for bacteria) to make exocellular cellulose fibrils that accumulate around the cells.

### The Family *LEGIONELLACEAE*

These rod-shaped bacteria require L-cysteine, iron salts, and activated powdered charcoal for growth (the charcoal destroys toxic hydrogen peroxide in the medium). All belong to a single genus, *Legionella*. The organisms are motile by polar or lateral flagella. They occur in surface water, thermally polluted lakes and streams, water from air-conditioning cooling towers and evaporative con-



densers, and in moist soil adjacent to a body of water. All species are opportunistic pathogens of humans, causing legionellosis.

### The Family NEISSERIACEAE

#### *Neisseria*

This family contains nonmotile rods and cocci that are catalase-positive and/or oxidase-positive. Examples are the genera *Neisseria* and *Acinetobacter*.

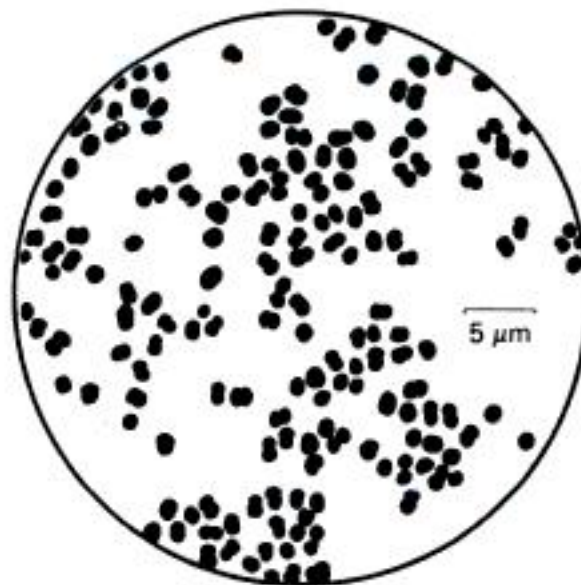
This genus has traditionally consisted of oxidase- and catalase-positive cocci that occur most often in pairs with the adjacent sides flattened (Fig. 13-8). However, one rod-shaped species (*N. elongata*) is now included because of its genetic relatedness. The neisseriae are parasites that inhabit the mucous membranes of humans and animals. Two species are highly pathogenic for humans, e.g., *N. gonorrhoeae*, the causative agent of gonorrhea, and *N. meningitidis*, the causative agent of epidemic cerebrospinal meningitis.

#### *Acinetobacter*

These diplobacilli are catalase-positive but oxidase-negative. They are saprophytes that occur in soil, water, and sewage, but they are also opportunistic human pathogens that can cause a variety of infections, particularly in hospitalized patients.

### Other Genera of Aerobic Gram-Negative Rods and Cocci Not Assigned to Any Family

Several genera are not assigned to any family, yet are included in this general section of *Bergey's Manual*. Some of these are described in Table 13-3.



**Figure 13-8.** Drawing of *Neisseria gonorrhoeae*, showing the characteristic diplococcus arrangement of the cells. (Erwin F. Lessel, illustrator.)

### FACULTATIVELY ANAEROBIC GRAM- NEGATIVE RODS

The organisms in this section form a very diverse group of straight or curved rods that can grow aerobically by respiring with oxygen and also under anaerobic conditions by fermenting various carbohydrates. Most genera are associated with animals or plants, but some occur in soil and water. The following are examples of some of the organisms included in this section.

### The Family ENTEROBACTERIACEAE

Some distinctive features of this family are:

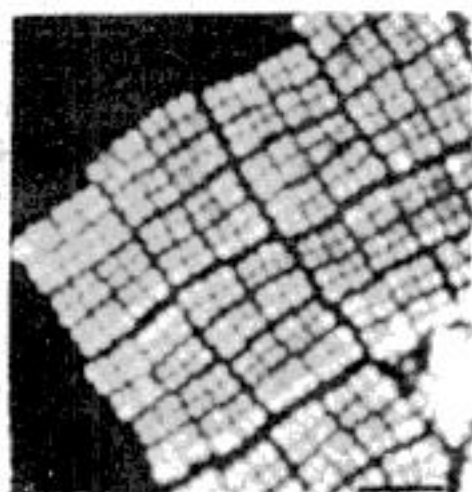
- 1 Cell diameter is 0.3 to 1.5  $\mu\text{m}$ .
- 2 Cell shape is that of a straight rod.



**Table 13-3.** Some Genera of Aerobic Gram-Negative Rods and Cocci Not Affiliated with Any Family

Genus	Shape	Flagella	Major Characteristics
<i>Beijerinckia</i> and <i>Derxia</i>	Rods	Peritrichous and polar, respectively	Tropical soil bacteria that can fix $N_2$ aerobically; <i>Derxia</i> can grow autotrophically with $H_2$ as the energy source
<i>Xanthobacter</i>	Rods	Usually none	Soil bacteria that fix $N_2$ under microaerophilic conditions; can grow autotrophically with $H_2$ as the energy source; form yellow colonies; cells stain Gram-positive or Gram-variable
<i>Thermus</i> and <i>Thermomicrobium</i>	Rods	None	Occur in hot springs; obligate thermophiles; optimum temperature is 70–75°C
<i>Alteromonas</i>	Straight or curved rods	Polar	Harmless marine organisms; require sea water for growth; oxidase-positive
<i>Flavobacterium</i>	Rods	None	Mainly saprophytes, widely distributed in nature; may often occur in hospital environments; form yellow to orange colonies; oxidase-positive; one species, <i>F. meningosepticum</i> , can cause a severe meningitis in newborn infants
<i>Alcaligenes</i>	Very short rods	Peritrichous	Occur in soil, freshwater, and marine environments but can be opportunistic human pathogens. Form non-pigmented colonies. Oxidase-positive. Some can grow autotrophically with $H_2$ as the energy source.
<i>Brucella</i>	Very short rods	None	Parasites and pathogens of animals. Three species are highly pathogenic for animals and humans, causing brucellosis.
<i>Bordetella</i>	Very short rods	None	Parasitic and pathogenic for various mammalian hosts. <i>B. pertussis</i> occurs only in humans and causes whooping cough.
<i>Francisella</i>	Very short rods	None	The major species, <i>F. tularensis</i> is a parasite of wild animals but can also cause tularemia in humans.
<i>Lampropedia</i>	Cocci, nearly cubical	None	Harmless saprophytes occurring in aquatic environments. Occur in distinctive, flat, square tablets of 16–64 cells (see Fig. 13-9).

- 3 Motility, if present, is by means of lateral flagella.
- 4 They are oxidase-negative.
- 5  $Na^+$  is not required or stimulatory for growth.
- 6 Cells contain a characteristic antigen, called the enterobacterial common antigen.
- 7 The organisms have simple nutritional requirements.



**Figure 13-9.** Negatively stained preparation of *Lampropedia hyalina* showing a sheet of actively growing tablets of cells. The bar indicates 5  $\mu m$ . (Courtesy of R. G. E. Murray, from *Bergey's Manual of Determinative Bacteriology*, 8th ed., Williams & Wilkins, Baltimore, 1974.)

**Table 13-4.** Typical Characteristics of Some Enterobacteriaceae

Property	<i>Escherichia</i>	<i>Shigella</i>	<i>Salmonella</i>	<i>Enterobacter</i>	<i>Serratia</i>	<i>Proteus</i>	<i>Yersinia</i>
Motility	d	-	+	+	+	+	-
Voges-Proskauer test	-	-	-	+	d	d	-
Indole from tryptophan	d	d	-	-	-	d	d
Hydrogen sulfide produced	-	-	+	-	-	d	-
Lysine decarboxylase	+	-	+	d	d	-	-
Gas from glucose	+	-	+	+	d	+	-
Acid from lactose	d	-	-	+	d	-	d
Urease	-	-	-	d	-	+	d
Phenylalanine deaminase	-	-	-	-	-	+	-
Deoxyribonuclease	-	-	-	-	+	-	-

+ = most or all species positive; - = most or all species negative; d = different reactions occur among species.

sugar-containing media are strongly acidified, acidic reactions from various sugars are used extensively to differentiate the genera and species (in contrast, for example, to the genus *Pseudomonas*). Some selected genera are listed below.

### *Escherichia*

(See Fig. 13-10.) The major species, *E. coli*, occurs in the lower portion of the intestine of humans and warm-blooded animals, where it is part of the normal flora. Some strains can cause gastroenteritis; others can cause urinary tract infections.

### *Shigella*

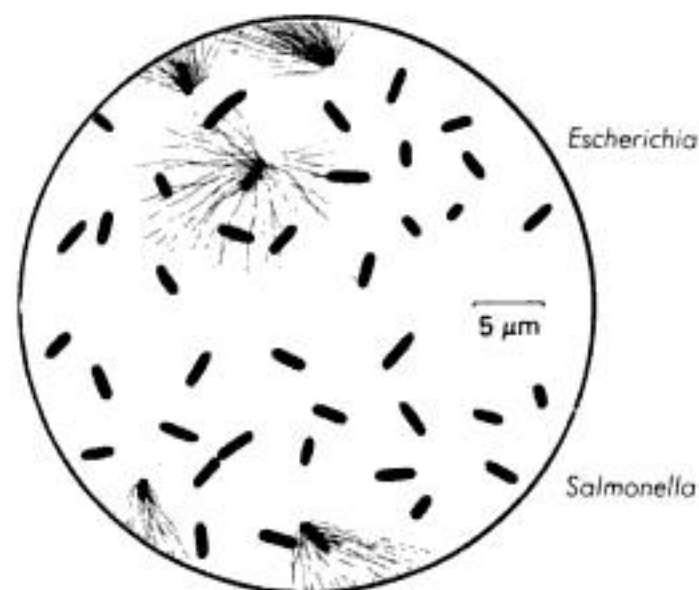
This genus is very closely related to *Escherichia* but differs in a few characteristics (Table 13-4). Moreover, all strains are pathogenic, causing bacillary dysentery in humans.

### *Salmonella*

(See Fig. 13-10.) This is a group of organisms that are closely related to one another and probably should be considered as a single species. All strains are pathogenic for humans, causing enteric fevers (such as typhoid and paratyphoid fevers), gastroenteritis, and septicemia; many strains also infect a variety of animals. Over 2,000 antigenic types of salmonellae occur.

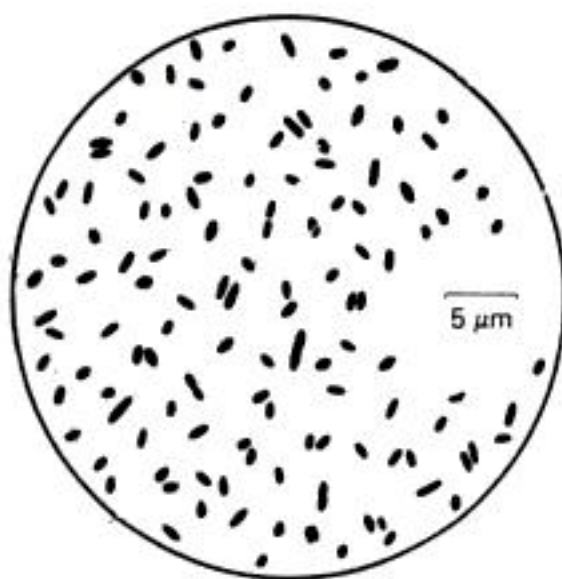
### *Enterobacter*

Unlike most other Enterobacteriaceae, *Enterobacter* species grow best at 30°C rather than at 37°C. They occur mainly in water, sewage, soil, meat, plants, and

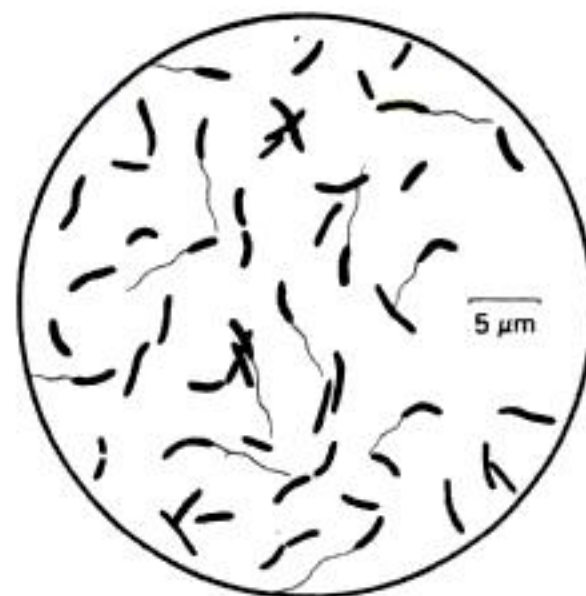


**Figure 13-10.** Drawing of cells of the genera *Escherichia* and *Salmonella*. The peritrichous flagella are not visible by ordinary staining. (Erwin F. Lessel, illustrator.)





**Figure 13-11.** Drawing of cells of *Yersinia pestis*, the causative agent of plague. (Erwin F. Lessel, illustrator.)



**Figure 13-12.** Drawing of cells of the genus *Vibrio*. The polar flagella are not visible by ordinary staining. (Erwin F. Lessel, illustrator.)

vegetables. Some species also occur in human and animal feces, and some can be opportunistic human pathogens.

### *Erwinia*

These bacteria differ from most other *Enterobacteriaceae* by being mainly associated with plants. They are often plant pathogens, causing diseases such as blights, cankers, die back, leaf spot, wilts, discoloration of plant tissues, and soft rots. They are seldom isolated from animals or humans.

### *Serratia*

The *serratiae* are widely distributed in soil, in water, and on plant surfaces. Many strains produce pink or red colonies. These organisms were once thought to be harmless; however, it is now clear that they can be opportunistic human pathogens and are particularly prone to infect hospitalized patients.

### *Proteus*

These organisms can swarm on agar media; that is, they spread over the plates in a thin film resulting from periodic cycles of migration. Such swarming often makes it difficult to obtain pure cultures of other bacteria from streak plates. *Proteus* strains occur in the intestine of humans and a wide variety of animals, in polluted waters, and in soil, and they can be opportunistic human pathogens. Like *E. coli*, *P. mirabilis* is one of the leading causes of urinary tract infections in humans.

### *Yersinia*

*Yersinia* are parasites of animals but can also cause infections in humans. For example, *Y. pestis* (see Fig. 13-11) is the causative agent of plague, and *Y. enterocolitica* is a frequent cause of gastroenteritis in children.

## The Family VIBRIONACEAE

Some distinctive features of this family are:

- 1 Cell diameter is 0.3 to 1.3  $\mu\text{m}$ .
- 2 Cell shape is that of curved or straight rods (Fig. 13-12).
- 3 The cells are motile by means of polar flagella.
- 4 They are usually oxidase-positive.

- 5  $\text{Na}^+$  is required or stimulatory for growth of some genera.
- 6 Cells do not contain the enterobacterial common antigen.
- 7 The organisms have simple nutritional requirements.

The Vibrionaceae occur in marine and freshwater environments or in association with animals living in those environments. Two genera are described below:

### *Vibrio*

These are distinguished from other members of the family by having flagella that are covered by a membrane (sheathed flagella). The organisms occur in aquatic habitats with a wide range of salinities. Some species can emit light of a blue-green color (bioluminescence), an oxygen-dependent reaction catalyzed by the enzyme luciferase. One such species, *V. fischeri*, occurs in the specialized luminous organs of certain deep-sea fishes. Most *Vibrio* species are harmless saprophytes, but some species are pathogenic; examples are *V. cholerae*, the causative agent of cholera in humans, *V. parahaemolyticus*, which causes gastroenteritis in humans, and *V. anguillarum*, which is a pathogen of marine fish and eels.

### *Aeromonas*

Cells are straight rods that have nonsheathed flagella. The organisms occur in fresh water sources and sewage. Some species are pathogenic for frogs and fish; e.g., *A. salmonicida* is the causative agent of furunculosis in salmon and trout.

### The Family PASTEURELLACEAE

Distinctive features of this family are:

- 1 The cell diameter is small (0.2 to 0.4  $\mu\text{m}$ ).
- 2 Cell shape is that of a straight rod.
- 3 The cells are nonmotile.
- 4 They are usually oxidase-positive.
- 5  $\text{Na}^+$  is not required or stimulatory for growth.
- 6 Cells do not contain the enterobacterial common antigen.
- 7 The organisms often have complex nutritional requirements.
- 8 The family occurs as parasites of vertebrates.

Some genera included in the family are described below:

### *Pasteurella*

These organisms are parasitic on the mucous membranes of the upper respiratory tract of mammals (rarely humans) and birds. The major pathogen is *P. multocida*, which causes hemorrhagic septicemia in cattle and fowl cholera in domestic and wild birds.

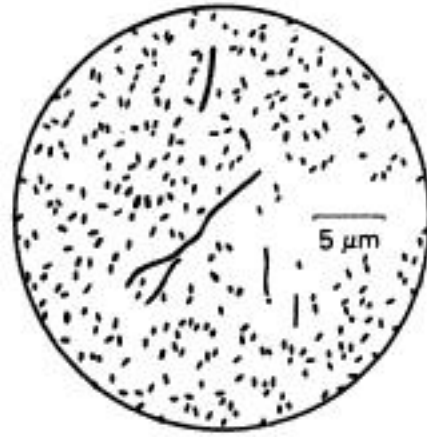
### *Haemophilus*

These bacteria are distinguished by unusual nutritional requirements: the X factor (heme, occurring in blood) and/or the V factor (the coenzyme nicotinamide adenine dinucleotide). *Haemophilus* species occur as parasites of the mucous membranes of humans and animals. Some are pathogenic for humans; for example, *H. influenzae* (see Fig. 13-13) is a leading cause of meningitis in children.

### *Actinobacillus*

These bacteria are also parasitic on mammals and birds. The organisms are only occasionally pathogenic for humans, but several species are pathogenic for





**Figure 13-13.** Drawing of cells of *Haemophilus influenzae*. (Erwin F. Lessel, illustrator.)

### Other Genera of Facultatively Anaerobic Gram-Negative Rods Not Assigned to Any Family

#### *Zymomonas*

animals, e.g., *A. lignieresii*, which causes granulomatous lesions in cattle and sheep, and *A. suis*, which causes septicemia, pneumonia, and arthritis in pigs.

Several genera of facultatively anaerobic rods are not assigned to any family but belong to this section of *Bergey's Manual*. A few examples are listed below.

#### *Chromobacterium*

These are saprophytic rods that occur in honey and as spoilage organisms in beer and cider. They are unusual because they form large amounts of ethanol from glucose.

These motile, rod-shaped organisms have the unusual property of forming violet colonies, due to a pigment called violacein. The species *C. violaceum* occurs as a saprophyte in soil and water but can occasionally cause infections of humans and other mammals.

#### *Gardnerella*

These nonmotile pleomorphic rods stain Gram-negative to Gram-variable. Whether they should be classified with Gram-negative bacteria based on studies of cell-wall ultrastructure and chemical composition is still inconclusive. The only species included in the genus, *G. vaginalis*, occurs in the human genitourinary tract and is a major cause of bacterial "nonspecific" vaginitis.

#### *Streptobacillus*

During cultivation of these pleomorphic rods, *L-phase variants* may occur spontaneously; these have a defective cell wall, are more or less spherical in shape, and form tiny "fried-egg" colonies similar to those formed by mycoplasmas (discussed later in this chapter). The single species of the genus, *S. moniliformis*, is a parasite of rats and causes one form of rat-bite fever in humans.

### ANAEROBIC, GRAM-NEGATIVE STRAIGHT, CURVED, AND HELICAL RODS

#### The Family BACTEROIDACEAE

The organisms in this section are placed within a single family, which is described as follows.

This family is a diverse assemblage of bacteria that exhibit the following features:

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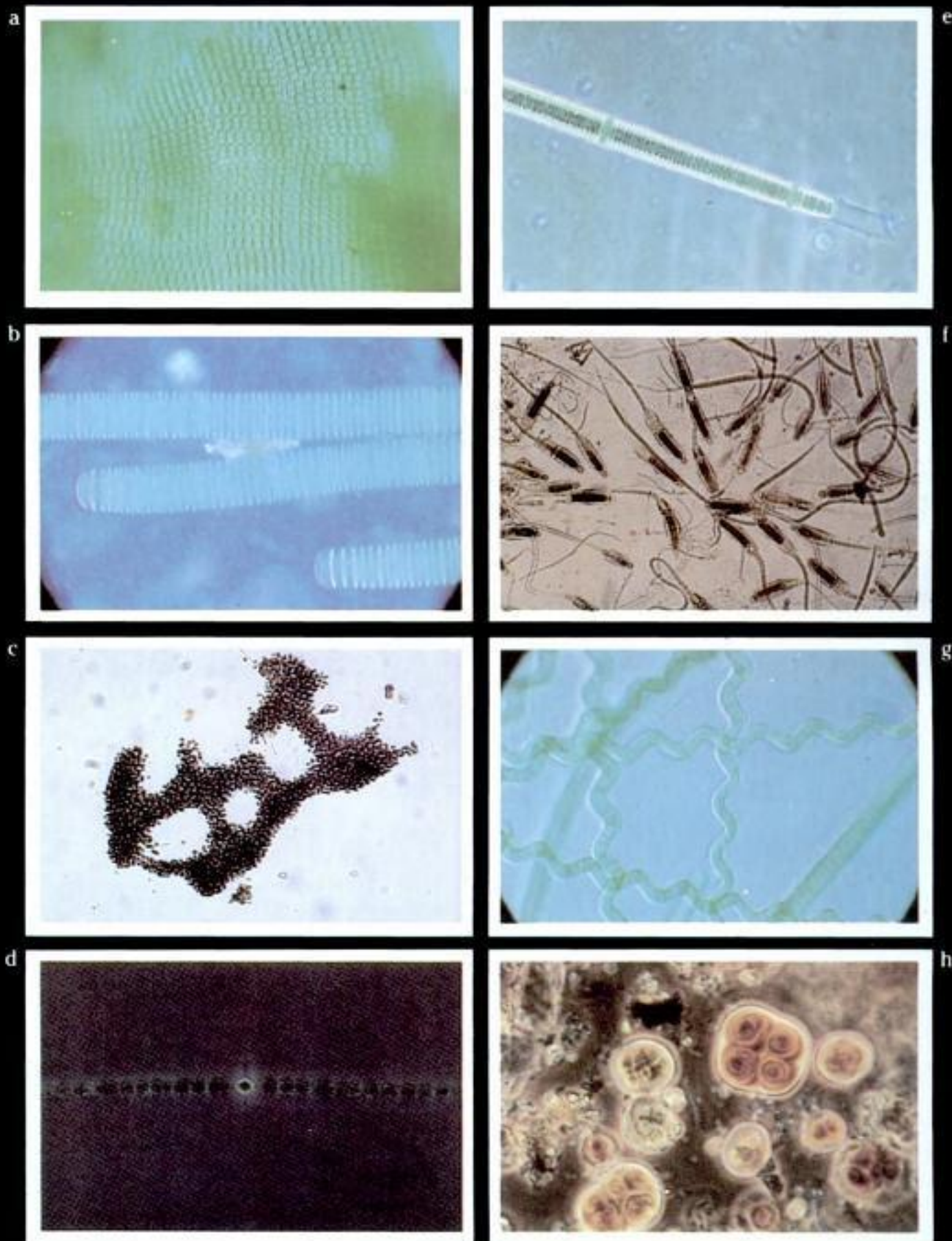


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**Cyanobacteria:**

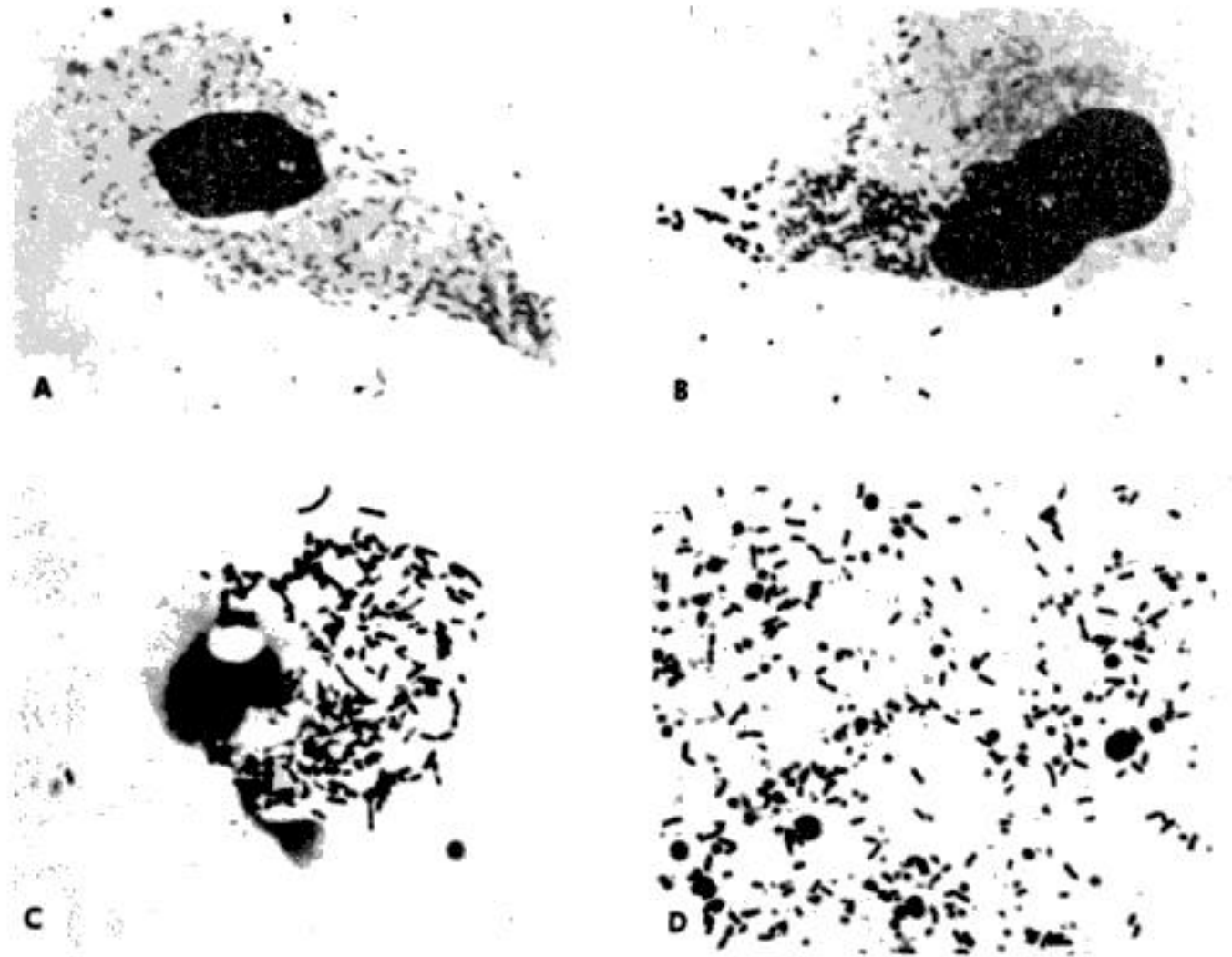
- a *Merismopedia glauca*.
- b *Oscillatoria* sp.
- c *Microcystis aeruginosa*.
- d *Anabaena planktonica*.
- e *Lyngbya aestuarii*.
- f *Gloeotrichia echinulata*.
- g *Arthrospira jennir*.
- h *Gloeocapsa repestis*.

(Courtesy of G. J. Schumacher, Harpur College, SUNY-Binghamton, New York.)





**Figure 13-16.** Photomicrographs of rickettsias grown under various conditions in the laboratory. (A) *Rickettsia akari* in smear of peritoneal scraping of infected laboratory mouse (X940). (B) *R. tsutsugamushi* in cytoplasm of infected cell (X940). (C) *R. prowazekii* in yolk-sac culture (X1,500). (D) *R. typhi* in yolk-sac culture (X1,000). (Courtesy of N. J. Kramis and the Rocky Mountain Laboratory, U.S. Public Health Service.)



- 1 Although the organisms are mainly parasites of humans and human body lice, they can be cultivated *in vitro* on laboratory media (e.g., a blood-based agar).
- 2 They grow *epicellularly* (i.e., on the surface of host cells) rather than in the cytoplasm or nucleus.

The single species of the genus, *R. quintana*, causes a louseborne disease, trench fever, in humans.

### *Coxiella*

This genus is distinguished by several unusual properties:

- 1 Growth occurs preferentially within membrane-bounded vacuoles of host cells rather than free in the cytoplasm or nucleus.
- 2 The organisms have an unusually high resistance to heat [may survive a temperature of 62°C (143°F) for 30 min], probably due to the occurrence of endospore-like structures in the cells.
- 3 Although transmission to vertebrates can occur via an arthropod vector, it occurs mainly by inhalation of airborne infectious dust. The organisms can also be acquired by drinking contaminated unpasteurized milk.

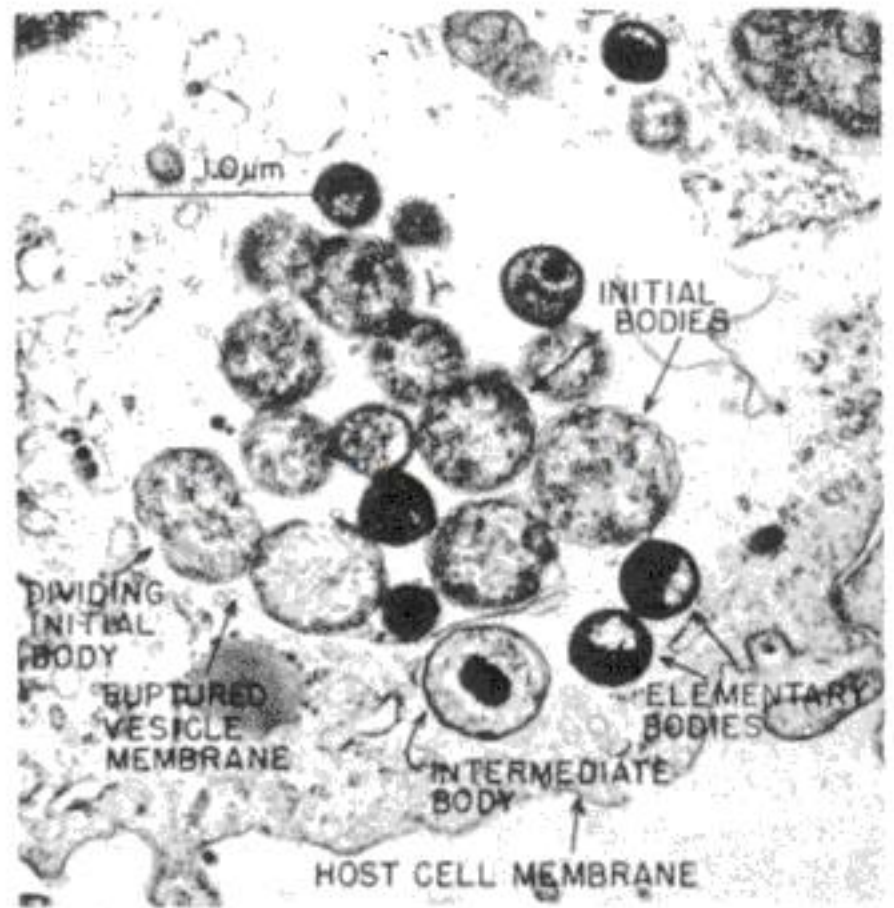
The single species of the genus, *C. burnetii*, is the causative agent of Q fever, a type of pneumonia.

### The Family *BARTONELLACEAE*

This family consists of parasites of the red blood cells of humans and other vertebrates. The organisms can be cultivated on nonliving laboratory media. The genus *Bartonella* causes Oroya fever in humans and is transmitted by biting



**Figure 13-17.** Electron micrograph of ultrathin section through a microcolony of *Chlamydia psittaci* in cytoplasm of a McCoy cell after 48-h incubation. The various developmental forms are labeled. The membrane of the vacuole containing the chlamydias has been ruptured, and the chlamydias are being released into the cytoplasm. The multilaminated nature of the wall of the elementary bodies and the double-unit membrane surrounding the reticulate (initial) bodies and the intermediate bodies (intermediate between the elementary and reticulate forms) are visible (X28,700). (Courtesy of Randall C. Cutlip, *Inf and Immun*, 1:500, 1970.)



flies that occur along the western slopes of the Andes mountains in South America.

### The Family ANAPLASMATACEAE

These organisms grow within or on erythrocytes or occur free in the plasma of various wild and domestic animals. None have been cultivated on nonliving laboratory media.

### The Order CHLAMYDIALES

This order contains the chlamydias, intracellular parasites that are distinguished from rickettsias by (1) an inability to make ATP (they have an absolute reliance on host cells for this compound and are sometimes termed “energy parasites”), and (2) occurrence of a characteristic developmental cycle. In the laboratory, chlamydias are cultivated in the yolk sac membrane of embryonated chicken eggs or in tissue cultures of mammalian cells, such as McCoy and HeLa cells.

Reproduction of chlamydias usually proceeds according to the following sequence.

- 1 An infectious small particle, or *elementary body* (see Fig. 13-17), having an electron-dense nucleoid, is taken into the host cell by phagocytosis.
- 2 The elementary body is enclosed within a membrane-bounded vacuole in the cytoplasm of the host cell.
- 3 Within the vacuole the elementary body is reorganized into a *reticulate body* (also termed *initial body*), which is two or three times the size of the elementary body and contains a less dense arrangement of nucleoid material (see Fig. 13-17). The reticulate body is not infectious (i.e., when cells are disrupted at this stage of chlamydial development the reticulate bodies that are liberated cannot infect other host cells).
- 4 The reticulate body undergoes *binary fission* until a number of reticulate bodies are formed, which then undergo reorganization into elementary bodies. This



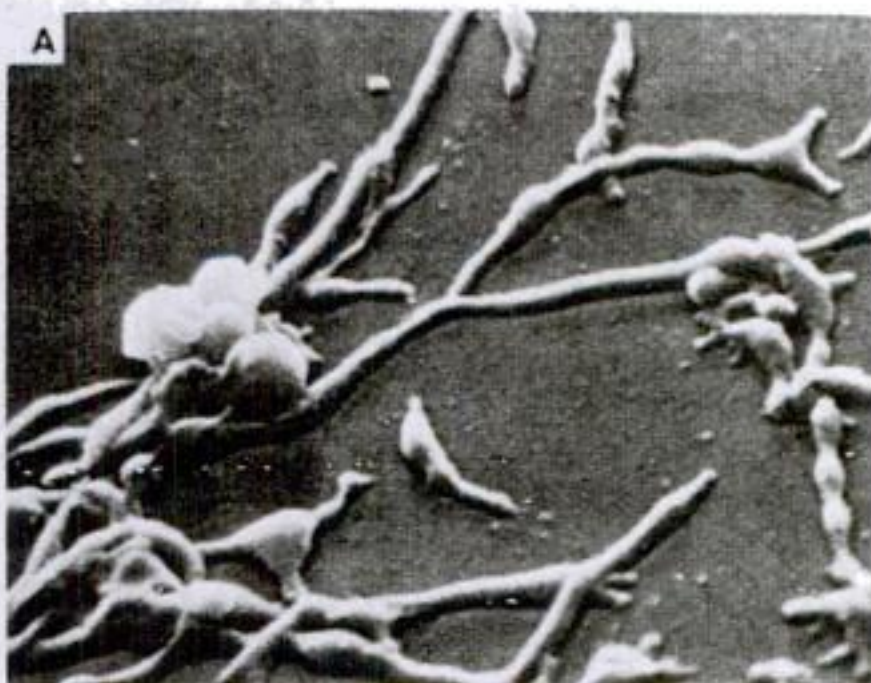
- aggregate of reticulate bodies and elementary bodies within the vacuole of the host cell forms a *host cell inclusion* which can be seen by light microscopy.
- 5 The progeny elementary bodies are then liberated from the host cell and proceed to infect other cells.

All chlamydias belong to a single family, *CHLAMYDIACEAE*, which contains a single genus, *Chlamydia*. Some strains of *C. trachomatis* cause a type of keratoconjunctivitis, trachoma, that often results in blindness. Other strains cause nongonococcal urethritis, which is the most prevalent sexually transmitted disease in the United States today. Still other strains cause the sexually transmitted disease lymphogranuloma venereum. The species *C. psittaci* is mainly a pathogen of birds and domestic and wild mammals but can also cause psittacosis in humans.

## THE MYCOPLASMAS

The mycoplasmas are distinguished by their lack of a cell wall, the outer boundary of the cells being the cytoplasmic membrane. As a result, the cells have plasticity and can assume many different shapes ranging from spheres to branched filaments (Fig. 13-18A). The plasticity allows many of the cells to pass through bacteriological filters even though the smallest cells are about 0.3  $\mu\text{m}$  in diameter. They are susceptible to lysis by osmotic shock caused by sudden dilution of the medium with water. Because of the lack of a cell wall, mycoplasmas are not inhibited by even high levels of penicillin; however, they can be inhibited by antibiotics that affect protein synthesis, such as tetracyclines or chloramphenicol. Mycoplasmas can be cultivated in vitro on nonliving media (of rich composition) as facultative anaerobes or obligate anaerobes. They have

**Figure 13-18.** Mycoplasma cells and colonies. (A) Scanning electron micrograph of *Mycoplasma pneumoniae* from a 6-day culture showing irregular forms, crossing filaments, and piling up of spherical organisms probably representing an early stage of colony formation (X27,600). (Courtesy of G. Biberfeld and P. Biberfeld, *J Bacteriol*, **102**:855, 1970.) (B) Colonies of *M. molare* showing the typical "fried-egg" appearance (X35). (Courtesy of S. Rosendal and *Int J Syst Bacteriol* **24**:125, 1974.)





genomes that are about one-fifth to one-half the size of those of other bacteria capable of growth on nonliving media, which explains why these organisms have complex nutritional requirements and limited biosynthetic abilities. Colonies on agar plates are usually tiny and require observation by means of a low-power microscope. The colonies are embedded in the agar surface and usually have a characteristic fried-egg appearance (Fig. 13-18B).

Mycoplasmas differ from the "L-phase variants" that can develop from other bacteria. Such variants are osmotically fragile, cell-wall-defective forms that may occur spontaneously (as in the genus *Streptobacillus*) or as the result of continuous exposure to sublethal levels of penicillin. They form fried-egg colonies resembling those of mycoplasmas. However, L-phase variants are derived from walled bacteria and can usually revert to the normal walled bacterial form (e.g., when penicillin is removed), whereas mycoplasmas do not give rise to walled forms. Moreover, penicillin-binding proteins and peptidoglycan precursors can be demonstrated in the membranes of L-phase variants but not in the membranes of mycoplasmas.

At present, mycoplasmas are placed in the taxonomic class *MOLLICUTES*, which contains the single order *MYCOPLASMATALES*. Three families are included in this order.

#### The Family *MYCOPLASMATACEAE*

These mycoplasmas are parasites of the mucous membranes and joints of humans or animals and require cholesterol for growth. Many species of the genus *Mycoplasma* are pathogenic for animals; the species *M. pneumoniae* has the most significance for humans and is the causative agent of primary atypical pneumonia. Members of the genus *Ureaplasma* require urea for growth and cause urethritis in humans, pneumonia in cattle, and urogenital disease in cattle and other animal species.

#### The Family *STREPTOMYCEACEAE*

These mycoplasmas do not require cholesterol for growth. They are widely distributed in vertebrates, in sewage and soil, and possibly on plants. Their pathogenicity is unknown. Only a single genus, *Acholeplasma*, occurs.

#### The Family *SPIROPLASMATACEAE*

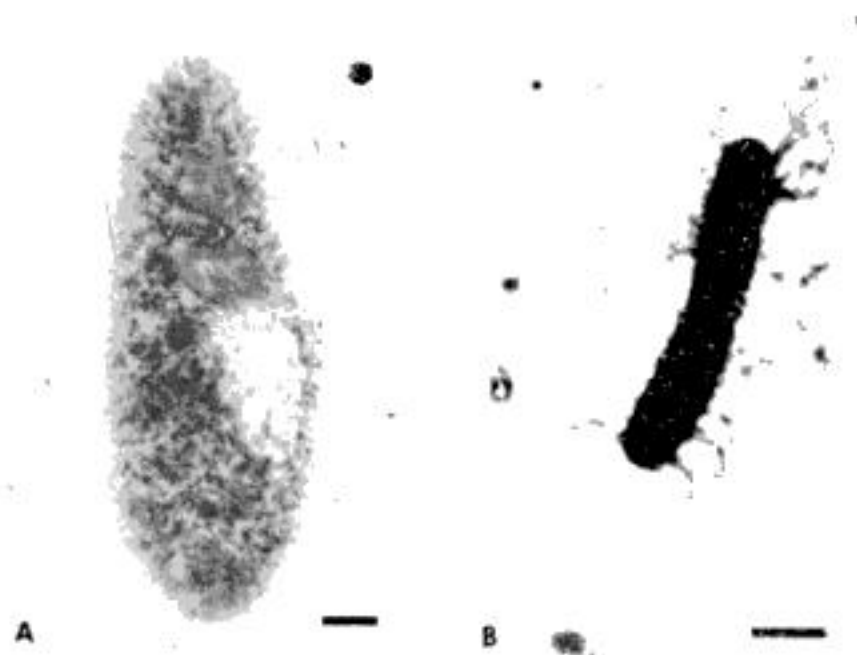
These organisms are unusual in that they are helical and exhibit a swimming motility. How a helical shape is maintained in the absence of a cell wall and how the cells can swim while lacking flagella is unknown. The family contains a single genus, *Spiroplasma*. The organisms are pathogenic for citrus and other plants. They can be isolated from plant fluids and plant surfaces and from arthropods that feed on plants.

#### ENDOSYMBIOSIS

A great variety of bacteria-like forms have been observed within the cells of protozoa, insects, fungi, sponges, coelenterates, helminths, and annelids. Most of these bacteria-like forms have not been cultivated in the laboratory, and information about them has generally been restricted to observations made with the light microscope or electron microscope. Most endosymbionts do not harm their hosts; indeed, many appear to be beneficial or even necessary for the growth and development of their hosts. This is suggested, for example, by the inability of certain insects to develop without vitamin supplements after being deprived of their endosymbionts by treatment with antibiotics or other means.



**Figure 13-19.** (A) Osmium-lacto-orcein preparation of the protozoan *Paramecium tetraurelia* bearing ensymbiont *Lyticum flagellatum*. The numerous black rods throughout the cytoplasm are the endosymbionts. Bar indicates 20  $\mu\text{m}$ . (B) *L. flagellatum* separated from its protozoan host, showing the peritrichous flagella; negatively stained with phosphotungstic acid. Bar indicates 1  $\mu\text{m}$ . (Courtesy of J. R. Preer, Jr., L. B. Preer, and A. Jurand, *Bacteriol Rev* 38:113, 1974.)



The greatest amount of information has been obtained for endosymbionts of protozoa, even to the point of classifying many of them by separating their DNA from that of their host cells and performing base composition and homology experiments with the DNA. Indeed, many of the protozoan endosymbionts now bear formal genus and species names. For example, *Lyticum flagellatum* is an endosymbiont carried by certain strains of the protozoan *Paramecium tetraurelia* (see Fig. 13-19). One function of *L. flagellatum* is to synthesize the vitamin folic acid for its host; symbiont-free lines of the same strains of the protozoan need to be supplied with this vitamin. Another function of *L. flagellatum* is to produce a toxin that is liberated into the culture medium: when an endosymbiont-bearing strain of *P. tetraurelia* (called a killer strain) is mixed with certain strains lacking it (called sensitive strains), the latter protozoa are rapidly killed and lysed. The endosymbiont-bearing strains are resistant to this toxin.

## QUESTIONS

- 1 In what way do spirochetes differ from other bacteria? What combination of characteristics sets them apart?
- 2 Which genera of Gram-negative bacteria are associated with plants as nitrogen fixers? As plant pathogens?
- 3 What general kinds of Gram-negative bacteria (i.e., aerobes, facultative anaerobes, or anaerobes) are associated with the ability to grow autotrophically with  $\text{H}_2$  as the energy source? With the ability to use methane gas as a carbon source?
- 4 List four genera of Gram-negative bacteria that produce distinctive pigments.
- 5 How are various sugars and other carbon sources used in the laboratory differentiation of *Pseudomonas* species? How does this differ from the way sugars are used to differentiate the genera of *Enterobacteriaceae*?
- 6 On what bases are the genera of the family *Bacteroidaceae* differentiated?
- 7 What is an opportunistic pathogen? List four Gram-negative bacteria that are opportunistic pathogens.
- 8 In what type of environment is one most likely to find (a) *Aquaspirillum*, (b) *Escherichia coli*, (c) *Thermus*, (d) *Erwinia*, (e) *Vibrio fischeri*, (f) *Zymomonas*?



- 9 List two anaerobic genera whose members obtain energy by respiration rather than by fermentation.
- 10 What are the major differences between rickettsias and chlamydias? Between mycoplasmas and other bacteria? Between *Mycoplasma* and *Acholeplasma*?
- 11 How are rickettsias generally transmitted to humans? How does this differ from the way in which Q fever is transmitted?
- 12 How are rickettsias and chlamydias cultured in the laboratory? In what way can *Rochalimaea quintana* be cultured that differs from the methods used for *Rickettsia* and *Chlamydia*?
- 13 What agent could you add to a growth medium for mycoplasmas that would make the medium selective for these organisms? Explain the basis for your answer.
- 14 What functions do endosymbionts serve for their hosts? How might endosymbionts have originated?

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## Chapter 14

# The World of Bacteria II: “Ordinary” Gram-Positive Bacteria

### OUTLINE *Bergey's Manual of Systematic Bacteriology, Volume 2*

#### **Gram-Positive Cocci**

Aerobic/Facultatively Anaerobic Cocci • Aerotolerant Fermentative Cocci • Anaerobic Gram-Positive Cocci

#### **Endospore-Forming Gram-Positive Bacteria**

Aerobic/Facultatively Anaerobic Sporeforming Rods and Cocci • Anaerobic Sporeforming Rods

#### **Nonsporeforming Gram-Positive Rods of Regular Shape**

#### **Nonsporeforming Gram-Positive Rods of Irregular Shape**

Aerobic/Facultatively Anaerobic Nonfilamentous Rods • Aerobic/Facultatively Anaerobic Branched Filamentous Rods • Anaerobic Nonfilamentous or Filamentous Rods

#### **Mycobacteria**

#### **Nocardioforms**

Like Volume 1 of *Bergey's Manual*, Volume 2 includes many of the familiar or “ordinary” chemoheterotrophic bacteria; however, the organisms are Gram-positive rather than Gram-negative. Most have a simple morphology and none form prosthecae, sheaths, or cysts. Some genera form heat-resistant endospores. Although most of the organisms have a regular, uniform shape, some are pleomorphic. Most of the organisms occur singly, in pairs, or in chains, but some are arranged in trichomes and others form branching hyphae. Motility, if present, is by means of flagella. In general, reproduction occurs mainly by transverse binary fission; however, multiplication by fragmentation or by conidiospore production can occur in several genera. Saprophytes and parasites are included; some of the parasitic organisms can be highly pathogenic for humans, animals, or plants. In this chapter we shall describe briefly some of the organisms included in the second volume of *Bergey's Manual*.

## BERGEY'S MANUAL OF SYSTEMATIC BACTERI- OLOGY, VOLUME 2

As in Chap. 13, the arrangement of the bacteria continues to be based primarily on practical considerations rather than on phylogenetic relationships. The organisms are divided into major sections, each bearing a descriptive common name. The major sections of Volume 2 are listed in Table 14-1. Formal taxonomic names are emphasized mainly at the family, genus, and species level of classification. In this chapter we shall indicate the great diversity of the organisms included in Volume 2 of *Bergey's Manual* by highlighting their important characteristics.

### GRAM-POSITIVE COCCI

#### Aerobic/Facultatively Anaerobic Cocci

All the cocci in this group have the following features

- 1 They possess cytochromes.
- 2 They are able to respire with oxygen, i.e., have an oxidative type of metabolism.
- 3 Some can also obtain energy under anaerobic conditions by fermentation (have a fermentative type of metabolism).

The organisms have been placed in two families. Members of the family *DEINOCOCCACEAE* exhibit the following characteristics:

- 1 The cocci occur mainly in tetrads or cubical packets.

**Table 14-1.** Gram-Positive  
Bacteria Included in  
*Bergey's Manual*, Volume 2

Section	Other Major Characteristics
GRAM-POSITIVE COCCI	May have a strictly respiratory type of metabolism, a respiratory plus a fermentative metabolism, or a strictly fermentative metabolism; in the latter category they may be able to grow in air (aerotolerant), or they may be anaerobic
ENDOSPORE-FORMING GRAM-POSITIVE BACTERIA	Mainly rod-shaped, but some are cocci; range from aerobic to facultatively anaerobic to anaerobic; most of the anaerobes live by fermentation, but some respire anaerobically with sulfate
NONSPOREFORMING GRAM-POSITIVE RODS OF REGULAR SHAPE	The cells have a uniform appearance without swellings, branching, or other types of variation; some occur in characteristic trichomes; aerobes, facultative anaerobes, or aerotolerant anaerobes are included
NONSPOREFORMING GRAM-POSITIVE RODS OF IRREGULAR SHAPE	The cells may exhibit swellings, Y or V shapes, rod/coccus cycles, or other deviations from a uniform morphology; some are filamentous during at least some stage of their growth; aerobic, facultatively anaerobic, and anaerobic genera are included
MYCOBACTERIA	Aerobic, slightly curved or straight rods which sometimes show branching; stain acid-fast
NOCARDIOFORMS	Aerobic organisms that tend to form a substrate mycelium and sometimes an aerial mycelium; the hyphae fragment into rod-shaped or coccoid elements; conidiospores may develop from the aerial hyphae



- 2 The organisms have an unusually high resistance to gamma and ultraviolet radiation.

The family contains a single genus, *Deinococcus*, which forms red colonies. The radiation resistance of the genus is reflected by the name of one of the species, *D. radiodurans*. The organisms can often be isolated as spoilage agents from foods preserved by treatment with ionizing radiation.

The family *MICROCOCCACEAE* exhibits the following features:

- 1 The cocci occur mainly in clusters, tetrads, or cubical packets of eight cells.
- 2 The cells do not exhibit any unusual resistance to gamma and ultraviolet radiation.

Three of the genera included in the family are described below.

### *Micrococcus*

These nonmotile cocci are aerobic, oxidative, and are catalase-positive. Their colonies may be red, orange, yellow, or nonpigmented. Micrococci are harmless saprophytes occurring in soil and freshwater, but they can also be found on the skin of humans and animals.

### *Planococcus*

These organisms are also aerobic, oxidative, catalase-positive cocci; however, the cells are motile and possess one to three flagella. The colonies are yellow-brown. Planococci are harmless saprophytes that occur in marine environments.

### *Staphylococcus*

See Fig. 14-1. Staphylococci are nonmotile cocci that are catalase-positive and facultatively anaerobic, having both an oxidative and a fermentative type of metabolism. They are parasites, occurring on the skin and mucous membranes of humans and warm-blooded animals. The major pathogenic species is *S. aureus*, which can cause boils, abscesses, wound infections, postoperative infections, toxic shock syndrome, and food poisoning in humans, and infections in animals, such as mastitis in cattle. In the laboratory, *S. aureus* produces white to golden-colored colonies and is positive for the coagulase test (a test for the ability of bacteria to cause blood plasma to clot). *S. epidermidis* and *S. saprophyticus*, which are coagulase-negative, can cause wound infections, endocarditis, and urinary tract infections.

### **Aerotolerant Fermentative Cocci**

These cocci have the following characteristics:

- 1 They do not possess cytochromes.
- 2 They have only a fermentative type of metabolism and do not respire; yet they can grow anaerobically or aerobically.
- 3 The cells are arranged in pairs, chains, or tetrads.

Some representative genera are described below.

### *Streptococcus*

This genus has the following features:

- 1 The cells are arranged in pairs or chains (see Fig. 14-1).
- 2 They are catalase-negative.
- 3 The organisms are homofermentative, i.e., the predominant end product of sugar fermentation is lactic acid. (In the case of *Streptococcus*, it is the L(+) optical isomer of lactic acid.)



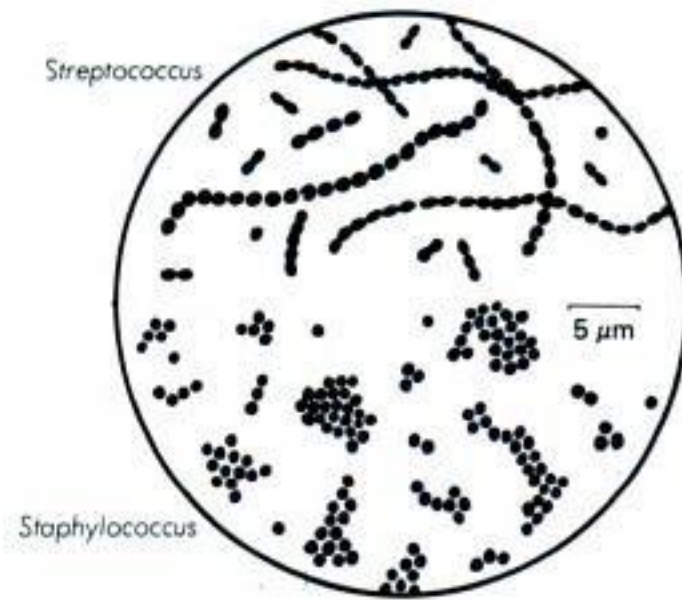
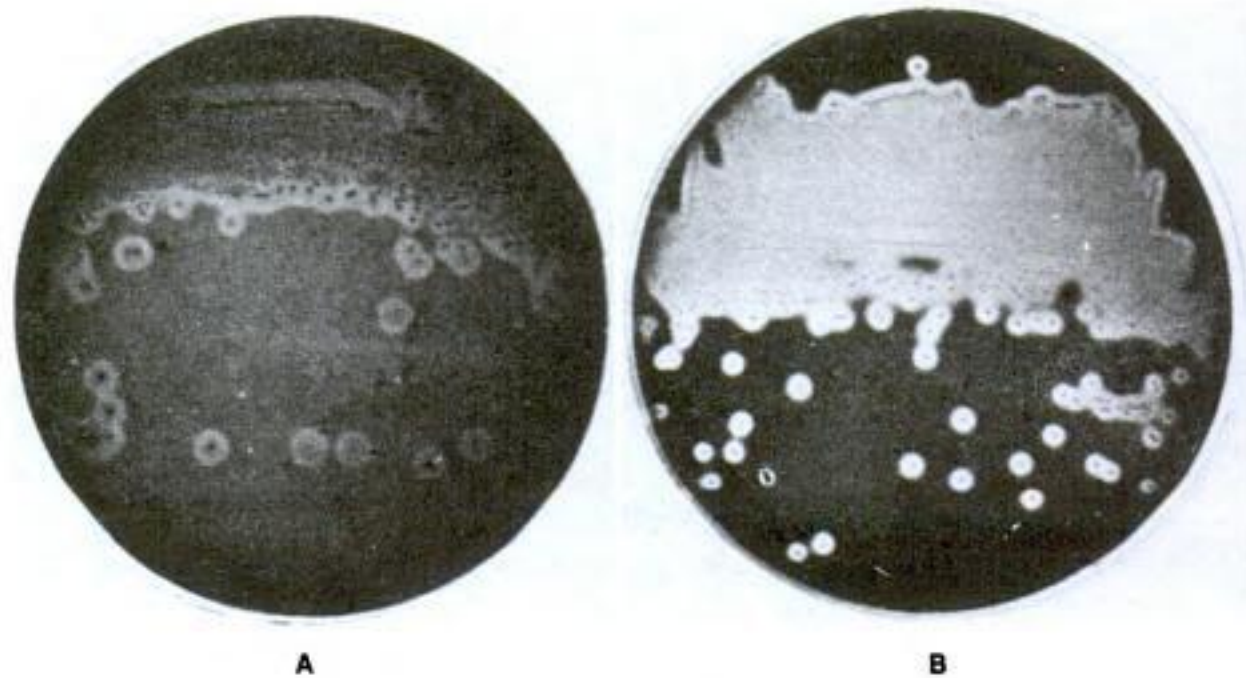


Figure 14-1. Drawing of the cells of *Staphylococcus* and *Streptococcus*. (Erwin F. Lessel, illustrator.)

**Figure 14-2.** (A)  $\alpha$ -hemolysis. Enzymes produced by some streptococci, such as *S. pneumoniae*, only partially hemolyze red blood cells of certain species of animals. Colonies on blood agar plates are surrounded by a greenish-colored zone which is due to the reduction of hemoglobin in the red blood cells to methemoglobin. (B)  $\beta$ -hemolysis. Enzymes produced by some streptococci, such as *S. pyogenes*, completely hemolyze red blood cells of certain species of animals. Colonies on blood agar plates are surrounded by a clear, colorless zone. (Courtesy of Liliane Therrien and E. C. S. Chan, McGill University.)



Although the genus is usually considered aerotolerant, some strains can tolerate only low levels of oxygen and some are anaerobic. Nutritional requirements are complex, including several amino acids and vitamins. The streptococci are divided into categories known as the *Lancefield* groups based on differences in their cell-wall polysaccharides.

Some streptococci are  $\beta$ -hemolytic on blood agar: the colonies are surrounded by a clear, colorless zone that indicates complete lysis of the erythrocytes (Fig. 14-2). Other streptococci are  $\alpha$ -hemolytic (colonies surrounded by a cloudy, colorless or greenish zone of partially lysed erythrocytes; see Fig. 14-2) or are nonhemolytic.

Most streptococci are parasites of humans and animals, and several species are pathogenic. There are many species of streptococci; a few examples follow.

*S. pyogenes* ( $\beta$ -hemolytic; Lancefield group A) is the most clinically important species. It causes streptococcal sore throat, scarlet fever, erysipelas, acute glomerulonephritis, rheumatic fever, and other human infections.

*S. mutans* (nonhemolytic; not placed in any Lancefield group) inhabits the human oral cavity and is the major causative agent of dental caries.

*S. faecalis* ( $\alpha$ -,  $\beta$ -, or nonhemolytic; Lancefield group D) occurs normally in



the intestinal tracts of humans and animals and is therefore called an "enterococcus"; it can be an opportunistic pathogen, causing urinary tract infections and endocarditis.

*S. lactis* and *S. cremoris* (Lancefield group N) are harmless contaminants of milk and dairy products; they cause rapid souring and curdling of milk, and because of this are widely used as "starter cultures" in the manufacture of buttermilk and cheeses.

*S. pneumoniae* ( $\alpha$ -hemolytic; not placed in any Lancefield group) is colloquially called the "pneumococcus" and has great clinical significance, causing nearly 70 percent of all cases of lobar pneumonia in humans.

### *Leuconostoc*

This genus has the following characteristics:

- 1 The cocci are arranged in pairs and chains.
- 2 They are catalase-negative.
- 3 The organisms are heterofermentative: they form  $\text{CO}_2$  and ethanol or acetic acid in addition to lactic acid; moreover, the lactic acid is of the D(-) type.

Leuconostocs are harmless saprophytes and are isolated from diverse sources such as grass, silage, grape leaves, sauerkraut, and spoiled food. They are often used in "starter cultures" for the manufacture of butter, buttermilk, and cheese because of their formation of the flavor compound diacetyl (2,3-butanedione) from citrate.

### *Pediococcus*

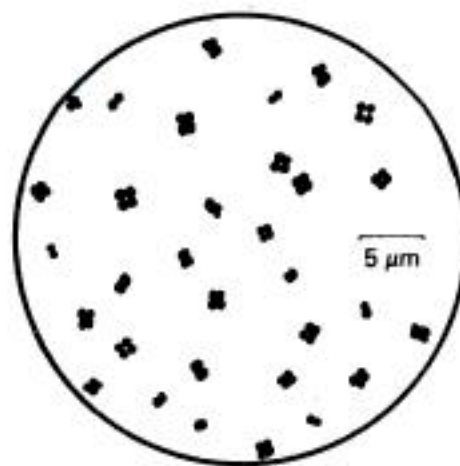
This genus has the following features:

- 1 Cocci occur in pairs and tetrads (see Fig. 14-3).
- 2 They are catalase-negative.
- 3 They exhibit a homolactic type of fermentation, forming optically inactive lactic acid, i.e., a mixture of the L(+) and D(-) types.

Pediococci are saprophytes and are particularly noted for their ability to form capsular material that causes beer to become ropy and viscous.

### Anaerobic Gram-Positive Cocci

These cocci have a fermentative type of metabolism. Some genera must be supplied with a fermentable carbohydrate in order to grow; others can ferment amino acids and do not require carbohydrates. Lactic acid, if formed, is not a major fermentation product (unlike the genus *Streptococcus*). Most genera form  $\text{CO}_2$ ,  $\text{H}_2$ , short-chain fatty acids, and in some cases ethanol or succinic acid.



**Figure 14-3.** Drawing of the cells of *Pediococcus cerevisiae*. (Erwin F. Lessel, illustrator.)

**Table 14-2.** Characteristics of Anaerobic Gram-Positive Cocci

Genus	Cell arrangement (cells)	Primary source of nutrients	Occurrence
<i>Peptococcus</i>	Pairs, clusters, tetrads, and short or long chains	Peptone or amino acids	Human intestine and respiratory tract; clinical specimens; tidal bay mud
<i>Peptostreptococcus</i>	Pairs and short or long chains	Peptone or amino acids	Human clinical specimens
<i>Ruminococcus</i>	Pairs and short or long chains	Carbohydrates	Bovine and ovine rumen; animal ceca
<i>Coprococcus</i>	Pairs and short or long chains	Carbohydrates	Human feces
<i>Sarcina</i>	Cubical packets of eight cells	Carbohydrates	Soil; mud; cereal grains; diseased human stomachs

Table 14-2 summarizes some of the characteristics for the various genera included in the group, and the morphological features of two of the genera are depicted in Fig. 14-4.

## ENDOSPORE-FORMING GRAM-POSITIVE BACTERIA

### Aerobic/Facultatively Anaerobic Species: Rods and Cocci

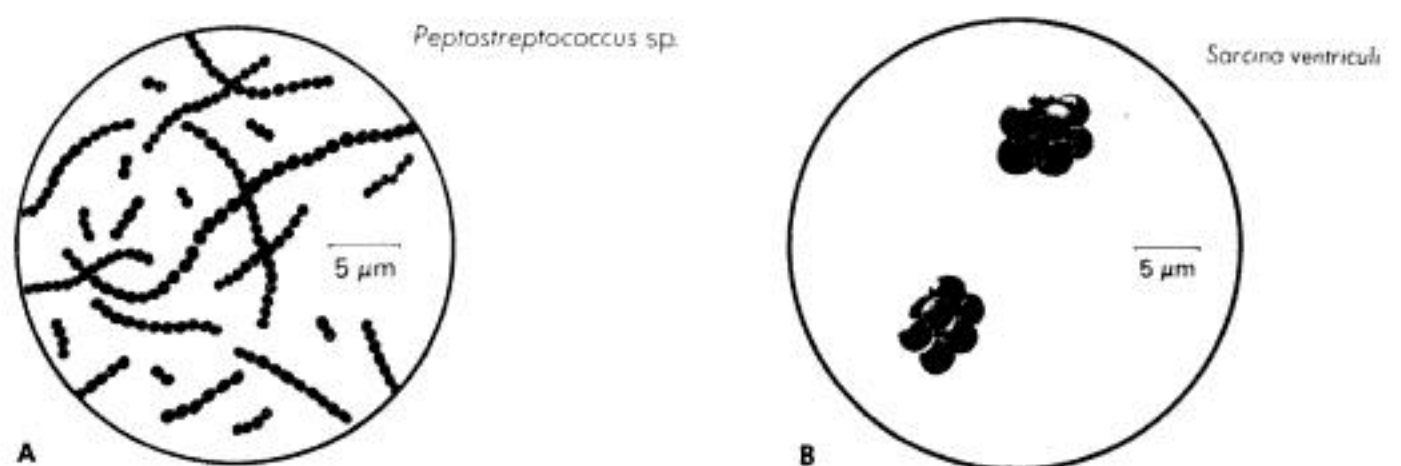
#### *Bacillus*

Most of the endospore-forming bacteria are rod-shaped, but some are cocci. The majority stain Gram-positive but some species stain Gram-negative. Motility, if present, is by means of peritrichous flagella. Some of the genera included in the group are described briefly here.

This genus contains rod-shaped bacteria. Most species are harmless saprophytes occurring in soil, freshwater, or sea water. Many form exocellular enzymes that hydrolyze proteins or complex polysaccharides, activities that are often important causes of food spoilage. Moreover, because of the heat resistance of the endospores, *Bacillus* species may survive milk pasteurization or inadequate heat treatment during canning of foods. A few examples of the many species are listed below.

*B. subtilis* and *B. cereus*. (See Fig. 5-29.) These common mesophilic sapro-

**Figure 14-4.** Anaerobic Gram-positive cocci. (A) Drawing of the cells of *Peptostreptococcus*. (B) Drawing of the cells of *Sarcina*. (Erwin F. Lessel, illustrator.)





phytes are widely distributed in nature. Both species produce exoenzymes that hydrolyze starch and casein. *B. cereus* can cause a type of food poisoning.

*B. stearothermophilus*. This is a thermophilic species having a minimum growth temperature of 30 to 45°C and a maximum of 65 to 75°C. The endospores are highly resistant to heat and, therefore, this species is one of those associated with spoilage of canned goods.

*B. polymyxa*. Unlike many other *Bacillus* species, *B. polymyxa* has the ability to form gas during sugar fermentation. Another unusual characteristic is its ability to fix  $N_2$  under anaerobic conditions.

*B. thuringiensis*. This species is noted for its pathogenicity for insects. Ingestion of the sporulated cultures of *B. thuringiensis* by larvae of *Lepidoptera* results in a paralytic disease. Other *Bacillus* species that are pathogenic for insects include *B. popilliae* ("milky disease" of Japanese beetle grubs) and certain strains of *B. thuringiensis* (lethal for mosquito larvae).

*B. anthracis*. This is the only *Bacillus* species that is highly pathogenic for animals and humans; it is the causative agent of anthrax.

### *Sporosarcina*

This genus contains cocci that are arranged in tetrads or cubical packets of eight cells (see Fig. 14-5). *Sporosarcinae* are widely distributed in fertile soil, where they play an active role in the decomposition of urea.

### Anaerobic Spore-forming Rods

#### *Clostridium*

The members of this genus have a fermentative type of metabolism. They are widely distributed in soil, in marine and freshwater anaerobic sediments, and in the intestinal tract of humans and animals. The species are differentiated on the basis of their proteolytic activity, various enzyme activities, acid production from carbohydrates, and the kinds and amounts of organic acid end products of fermentation (the latter being determined by gas chromatography). A few of the many species in the genus are listed below:

*C. botulinum* causes a severe and often fatal type of food poisoning known as botulism.

*C. tetani* is the causative agent of tetanus. The characteristic terminal spores formed by this species are illustrated in Fig. 5-29.

*C. perfringens* is the major causative agent of the wound infection known as gas gangrene. Some strains of *C. perfringens* (enterotoxigenic strains) can cause a type of food poisoning.

*C. difficile* causes pseudomembranous colitis, a severe disease of the bowel.

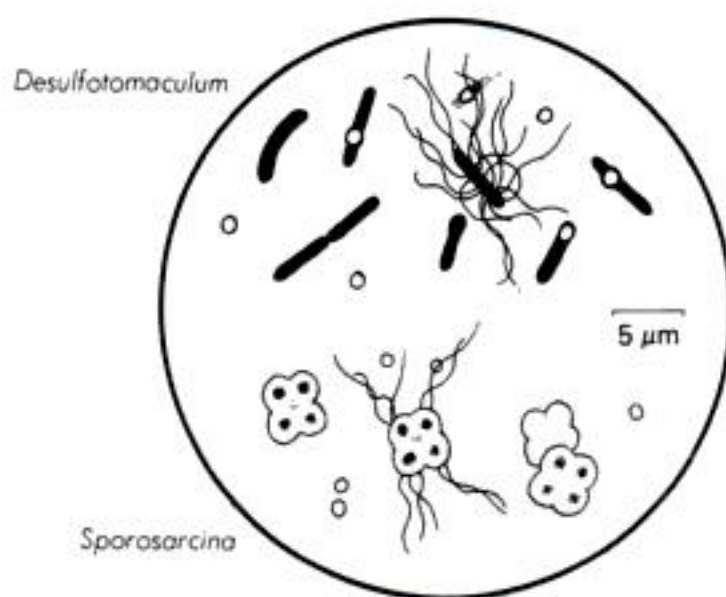
*C. thermosaccharolyticum* is thermophilic, growing optimally at 55°C (minimum temperature 45°C; maximum, 67°C). The spores are extremely heat-resistant, and this species is often able to survive inadequate heat treatment during canning of foods and subsequently can cause spoilage of canned goods.

*C. pasteurianum* is a mesophilic soil clostridium that is particularly noted for its ability to fix  $N_2$ .

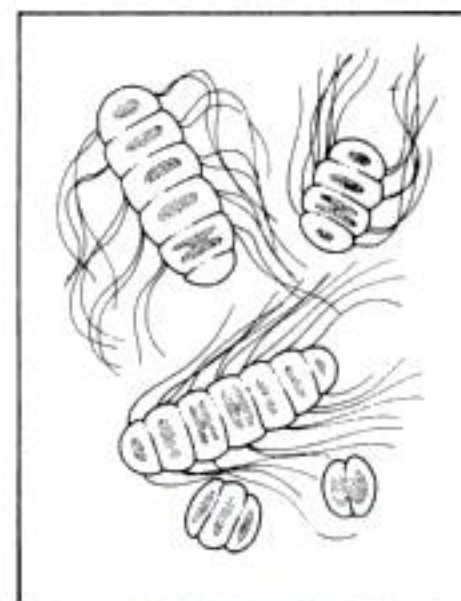
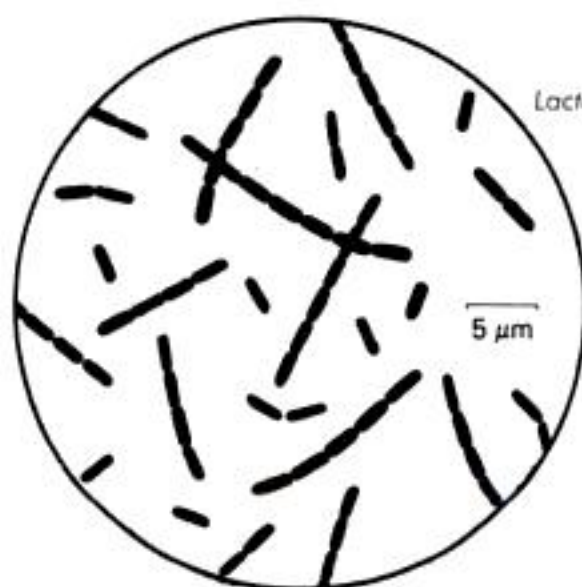
#### *Desulfotomaculum*

Unlike clostridia, members of this genus obtain energy by anaerobic respiration, with sulfate serving as the terminal electron acceptor and organic substrates such as lactic or pyruvic acid serving as the electron donors. Large amounts of  $H_2S$  are formed during growth. The organisms occur in soil, freshwater, intestines of insects, and the rumen of animals. See Fig. 14-5.

**Figure 14-5.** Drawing of the cells of *Sporosarcina* and *Desulfotomaculum*, showing the endospores and flagella. The flagella are not visible by ordinary staining. (Erwin F. Lessel, illustrator.)



**Figure 14-6.** (A) Drawing of the cells of *Lactobacillus*. (Erwin F. Lessel, illustrator.) (B) Sketch of *Caryophanon* showing trichomes 3 µm in diameter composed of disk-shaped cells, together with numerous peritrichous flagella. (Redrawn from K. A. Bisset, *Bacteria*, E. and S. Livingstone, Ltd., Edinburgh, 1952.)



### NONSPOREFORMING GRAM-POSITIVE RODS OF REGULAR SHAPE

### NONSPOREFORMING GRAM-POSITIVE RODS OF IRREGULAR SHAPE

### Aerobic/Facultatively Anaerobic Nonfilamentous Rods

#### *Corynebacterium*

This heterogeneous group is composed of harmless saprophytes as well as parasitic and pathogenic organisms. The cells range from long rods to very short rods as, for example, in the genus *Lactobacillus* (Fig. 14-6A). One genus, *Caryophanon*, is unusual in that it is composed of large, disk-shaped cells arranged in trichomes (Fig. 14-6B). Some characteristics of the various genera included in the group are indicated in Table 14-3.

This group contains a heterogeneous variety of bacteria, the few common features being:

- 1 Straight to slightly curved rods that exhibit swellings, club shapes, or other deviations from a uniform rod shape
- 2 An aerobic or facultatively anaerobic nature, being capable of a respiratory type of metabolism and in some instances also of a fermentative type of metabolism

Some examples of the genera included in this group follow.

This genus contains rod-shaped cells which are pleomorphic and frequently

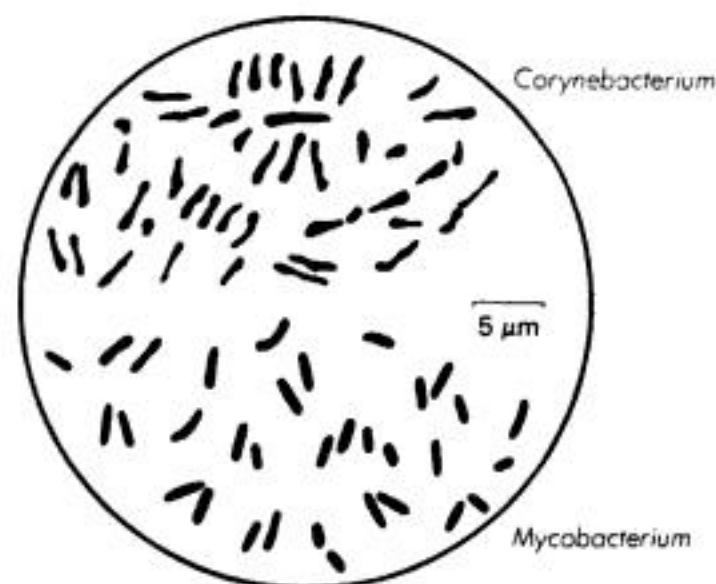


**Table 14-3.** Genera of Nonsporeforming Gram-Positive Rods of Regular Shape

Genus	Morphology and Motility	Oxygen Relationships	Catalase Test	Other Characteristics
<b><i>Lactobacillus</i></b>	Long to very short rods, often in chains; usually nonmotile	Strictly fermentative organisms, but can usually tolerate air; some strains are anaerobic	—	Large amounts of lactic acid formed; homo- or heterofermentative; occur as saprophytes in fermenting animal or plant products or as parasites in the mouth, vagina, and intestinal tract of humans and warm-blooded animals
<b><i>Listeria</i></b>	Very short rods, often in chains; motile by peritrichous flagella when grown at 25°C; few flagella are formed at 37°C	Aerobic to microaerophilic	+	The species <i>L. monocytogenes</i> is a parasite and pathogen of a wide variety of animals; in humans it causes meningitis in adults and prenatal or postnatal disease in infants
<b><i>Erysipelothrix</i></b>	Filament-forming rods; nonmotile	Aerobic	—	Parasitic on mammals, birds, and fish; causes erysipelas in swine and erysipeloid in humans
<b><i>Brocothrix</i></b>	Rods, often occurring in long, kinked filaments; nonmotile	Facultatively anaerobic	+	Best growth occurs at 20 to 22°C; none at 37°C; saprophytes, found in meat and meat products
<b><i>Renibacterium</i></b>	Short rods; nonmotile	Aerobic	+	Best growth occurs at 15 to 18°C; parasites of salmonid fishes, causing a kidney disease
<b><i>Kurthia</i></b>	Rods in chains; motile by peritrichous flagella	Aerobic	+	Harmless saprophytes occurring in meat and meat products and in animal dung
<b><i>Caryophanon</i></b>	Large disk-shaped cells arranged in trichomes; motile by peritrichous flagella	Aerobic	Not reported	The morphology is unusual and distinctive; saprophytic, occurring in ruminant dung

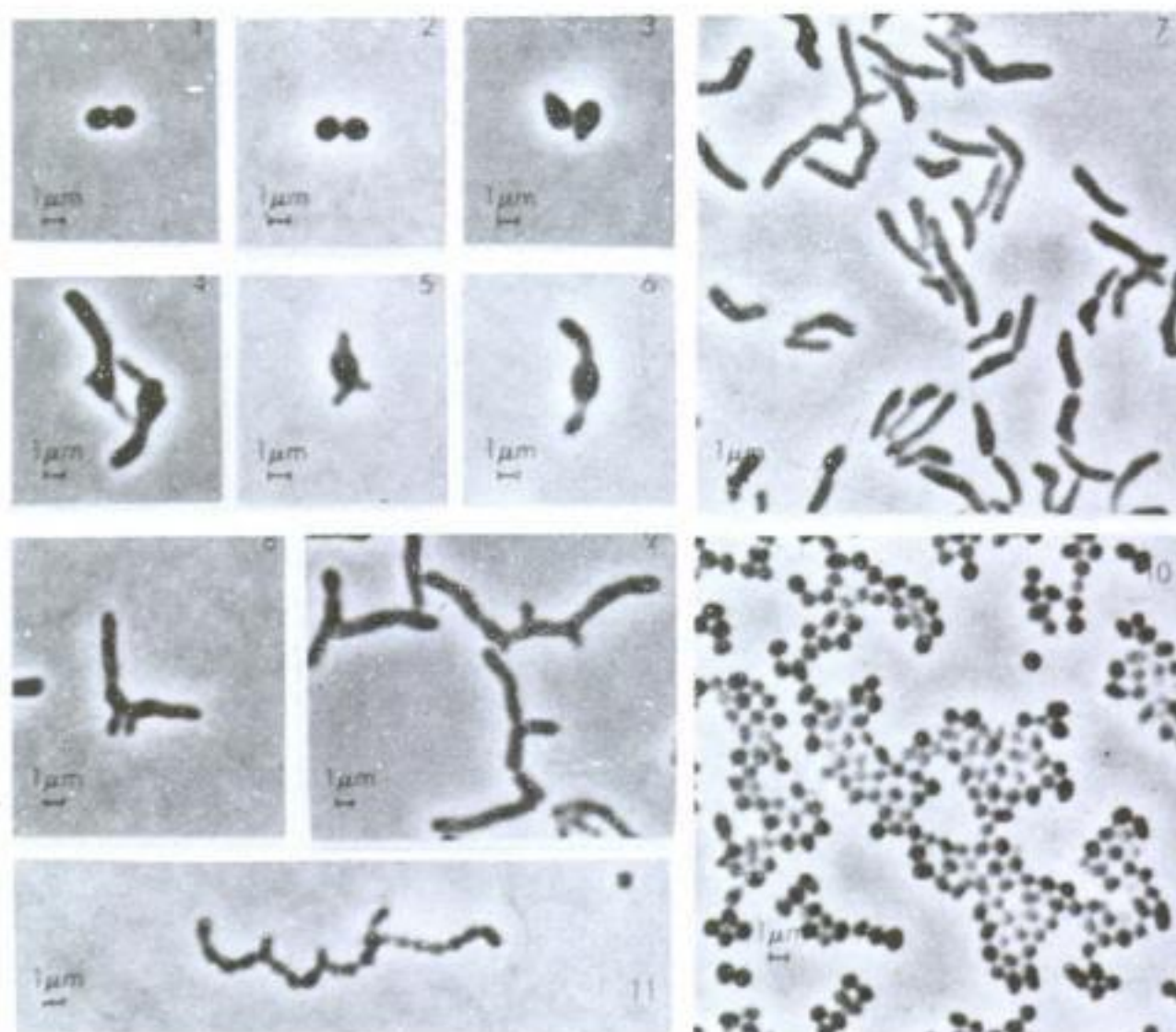
exhibit club-shaped swellings and a palisade arrangement (see Fig. 14-7). The cells accumulate intracellular volutin granules (metachromatic granules) which

**Figure 14-7.** Drawing of the cells of *Corynebacterium* and *Mycobacterium*. (Erwin F. Lessel, illustrator.)





**Figure 14-8.** Morphology of *Arthrobacter globiformis* at different times of growth and on different media. Insets 1, 2, 3, and 4, slide cultures grown on yeast extract–soil extract medium for 1.5, 4.25, 6, and 9.75 h, respectively; insets 7 and 8, after growth for 24 h. Insets 5 and 6, cultures grown on yeast extract–mineral salts medium for 5 h; inset 11, grown for 3 days. Insets 9 and 10, grown on yeast extract–peptone–soil extract medium for 11.5 h and 3 days. (Courtesy R. M. Keddie and Bergey's Manual of Determinative Bacteriology, 8th ed., Williams & Wilkins, Baltimore, 1974.)



stain reddish purple with dilute methylene blue. The cell walls contain mycolic acids (corynemycolic acids) containing 32 to 36 carbon atoms.

The corynebacteria are divided into three large groups: saprophytes occurring in soil and water, the animal or human parasites and pathogens, and the plant pathogens. Of the animal or human pathogens, the major species is *C. diphtheriae*, the causative agent of diphtheria in humans.

### *Arthrobacter*

This genus of saprophytic soil organisms is characterized by an unusual “rod-coccus” cycle. Cells in the log phase of growth are irregularly shaped rods that may show a tendency toward rudimentary branching. In contrast, cells in the stationary phase of growth are distinctly coccoid; when these are inoculated into fresh media, they give rise to rod-shaped cells (see Fig. 14-8).

### *Brevibacterium*

Like arthrobacters, brevibacteria exhibit a rod-coccus cycle. The only recognized species, *B. linens*, forms orange colonies and is salt-tolerant; its usual habitat is on the surface of certain cheeses such as brick and Limburger, where it produces proteolytic enzymes that aid in the cheese-ripening process.

### *Microbacterium*

These bacteria are small, slender, irregularly shaped rods that do not exhibit a rod-coccus cycle (although the rods become shorter in the stationary phase of growth). Microbacteria are saprophytes that occur in milk, in dairy products, and on dairy equipment.



**Cellulomonas**

This genus contains irregularly shaped rods that may be slightly filamentous and show rudimentary branching. No *Arthrobacter*-like rod-coccus cycle occurs, although a few cells in old cultures may be coccoid. The outstanding characteristic of the genus is the ability to degrade cellulose and to use it as a major carbon and energy source.

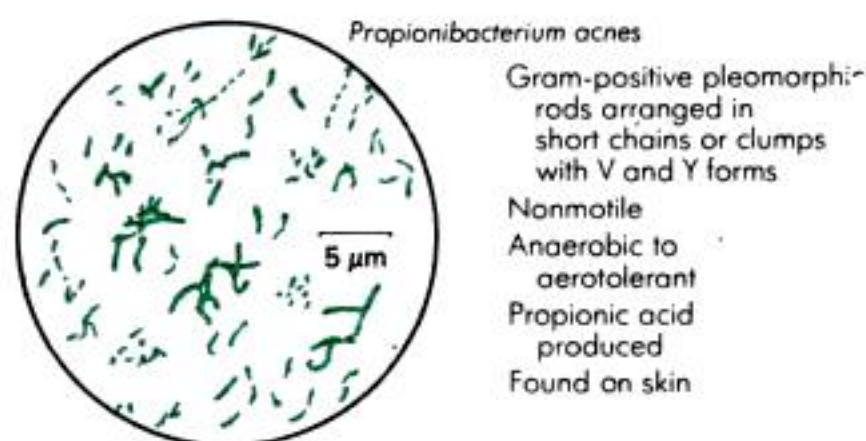
**Aerobic/Facultatively Anaerobic Branched Filamentous Rods**

The bacteria of this group form colonies which at first are microscopic in size (**microcolonies**) and contain branched filamentous cells. As the colonies develop to macroscopic size, many of the cells become diphtheroid (i.e., resemble *Corynebacteria*) or coccoid in shape. One genus, *Agromyces*, is microaerophilic to aerobic, catalase-negative, and is a saprophyte that occurs in soil. The genus *Arachnia* is facultatively anaerobic, catalase-negative, and parasitic and pathogenic for humans and animals, being one of the causative agents of actinomycosis. The genus *Rothia* is aerobic, catalase-positive, and a normal inhabitant of the human mouth.

**Anaerobic Nonfilamentous or Filamentous Rods**

The organisms of this group are either anaerobes or, if facultatively anaerobic, are preferentially anaerobic. They are differentiated by their morphology and by their fermentation end products as determined by gas chromatography. Table 14-4 indicates the characteristics of several genera included in the group, and the morphological features of two genera, *Propionibacterium* and *Actinomyces*, are depicted in Figs. 14-9 and 14-10.

**Figure 14-9.** (Right) Drawing of the cells of *Propionibacterium acnes*. (Erwin F. Lessel, illustrator.)



**Figure 14-10.** (Below) *Actinomyces israelii*. (A) Dark-field preparation showing V and Y forms. (B) Gram-stain preparation showing elongated filaments, branching, and irregular staining. (C) Gram-stain preparation showing mass of intertwined filaments. Some filaments are irregularly stained, and some have bulbous ends. (Approximately X800.) From J. M. Slack, S. Lendfried, and M. A. Gerencser, *J Bacteriol*, 97:873, 1969. By permission.)





## MYCOBACTERIA

This group of aerobic bacteria contains a single genus, *Mycobacterium*, which consists of slightly curved or straight rods (see Fig. 14-7) that may show branching. Mycolic acids having about 90 carbon atoms occur in the cell walls. A major characteristic of mycobacteria is that they are acid-fast; that is, once stained with aniline dyes, they are difficult to decolorize, even when treated with a mixture of acid and alcohol. Some species such as *M. phlei* and *M. smegmatis* are harmless saprophytes. Many species are pathogenic, for example, *M. tuberculosis*, the causative agent of tuberculosis in humans; *M. kansasii* and *M. intracellulare*, which cause noncontagious tuberculosis-like infections; *M. scrofulaceum*, which causes lymphadenitis in children; and *M. leprae*, the causative agent of leprosy.

## NOCARDIOFORMS

This group contains aerobic bacteria that produce a **substrate mycelium**, i.e., a mat of branching hyphae formed under the surface of the agar medium. This mycelium may range from rudimentary to extensively developed. The substrate mycelium usually fragments into rod-shaped or coccoid cells. Some genera also form an **aerial mycelium** that may give rise to conidiospores. Because the various genera of the group resemble the genus *Nocardia* with respect to their morphology and cell-wall composition, they are referred to as the **nocardioforms**. Several genera possess mycolic acids (nocardomycolic acids) in the cell walls; however, only the genus *Nocardia* contains acid-fast species.

The taxonomic placement of mycelium-forming bacteria, including the nocardioforms, is confused and controversial. Among the methods that have been used to establish various groupings are studies of the chemical composition of the cell walls. The walls of the nocardioform bacteria have the following characteristics, which define the type IV chemotype:

**Table 14-4.** Some Anaerobic, Irregularly Shaped, Nonsporeforming Gram-Positive Bacteria

Genus	Morphology	Organic Acids from Fermentation	Occurrence
<i>Propionibacterium</i>	Pleomorphic, nonmotile	Mainly propionic + acetic acids	Some species occur in dairy products; others are normal flora of human skin and of the intestines of humans and animals; <i>P. acnes</i> may be related to the skin disease acne vulgaris
<i>Eubacterium</i>	Pleomorphic, motile or nonmotile	Either butyric + other acids; acetic + formic; or no major acids	Human oral cavity, intestinal tract of humans and animals, infected tissues, soil, water, spoiled food; usually not pathogenic
<i>Actinomyces</i>	Initially, cells are filamentous with branching; eventually, diphtheroid cells predominate	Moderate amounts of acetic and sometimes formic, together with large amounts of succinic or lactic, or both	Oral cavity of humans and animals and human female genital tract; <i>A. israelii</i> and other species can cause human actinomycosis; <i>A. bovis</i> causes actinomycosis (lumpy jaw) in cattle
<i>Bifidobacterium</i>	Pleomorphic, nonmotile	Acetic and lactic acids	Intestinal tract of humans and animals; not known to be pathogenic



- 1 The peptidoglycan contains meso-diaminopimelic acid.
- 2 No glycine interpeptide bridges occur between the peptidoglycan chains.
- 3 The walls contain the sugars arabinose and galactose.

Other groups of mycelium-forming bacteria differ in these respects; for example, the walls of members of the genus *Streptomyces* (see Chap. 16) contain LL-diaminopimelic acid, glycine interpeptide bridges, and no distinctive sugars (type I chemotype).

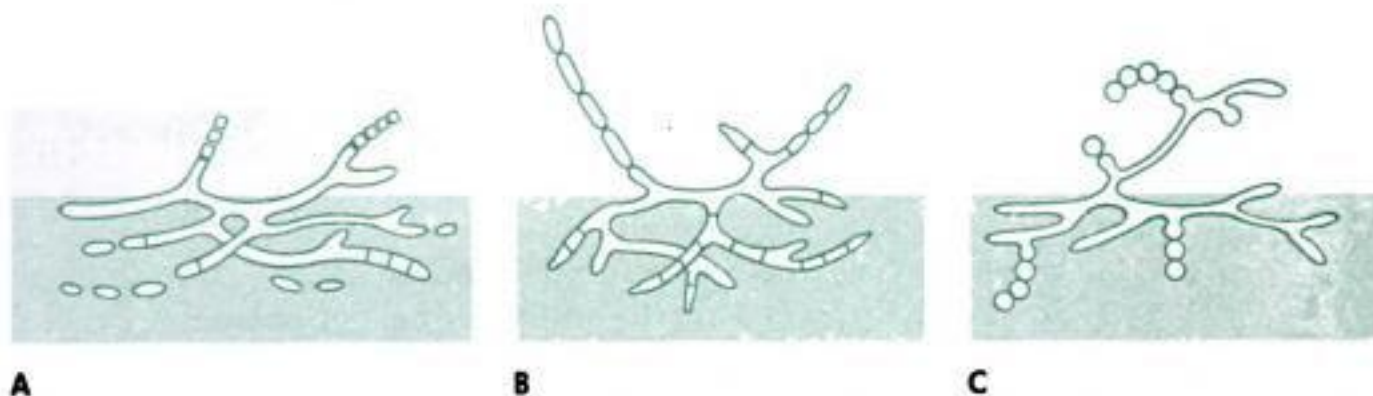
A few examples of the nocardioform group are described below.

### *Nocardia*

The morphological features of this genus are illustrated in Figs. 14-11 and 14-12A. Some nocardias form only a limited mycelium because the center of the colony undergoes early fragmentation into rod-shaped or coccoid cells. Other



**Figure 14-11.** *Nocardia asteroides*. Three different strains of the same species, showing variations in morphology. (X700) (Courtesy of Ruth E. Gordon.)



**Figure 14-12.** Schematic representation of the morphology of some nocardioform bacteria. Both substrate and aerial mycelia are illustrated. (A) *Nocardia*, showing fragmentation and aerial chains of spores. (B) *Pseudonocardia*, showing budding of the hyphae and aerial chains of cylindrical spores. (C) *Micropolyspora*, showing chains of round spores on both aerial and substrate mycelium.



nocardias have *delayed fragmentation* and are thus able to establish an extensive substrate mycelium, and sometimes an aerial mycelium that gives the surface of the colonies a dull, fuzzy appearance. The aerial hyphae of some species may bear chains of spores. Nocardias are saprophytes that are widely distributed in soil and water, but they can be opportunistic pathogens, causing nocardiosis and actinomycetoma in humans and animals.

### *Pseudonocardia*

This genus does not contain nocardomycolic acids, and, in contrast to the hyphae of nocardias, the hyphae of pseudonocardias grow by a distinctive budding process. A constriction occurs behind the hyphal tip; the tip elongates to form a new hyphal segment; this segment develops a constriction behind the tip, and in turn undergoes budding, etc. The aerial mycelium bears long chains of cylindrical conidiospores, which arise terminally or laterally from the hyphae (see Fig. 14-12B). Pseudonocardias occur in soil and in fresh or rotten manure.

This genus forms an extensive aerial mycelium. The hyphae of both the substrate and aerial mycelia bear short chains of 1 to 20 round conidiospores (see Fig. 14-12C). The spores occur in moldy hay or silage or in the air of farm buildings and can be inhaled into the lungs; however, they are apparently not pathogenic. Nocardomycolic acids occur in the walls of some *Micropolyspora* species.

## QUESTIONS

- 1 How is *Micrococcus* distinguished from *Staphylococcus*? From *Deinococcus*? From *Planococcus*?
- 2 How is *Leuconostoc* distinguished from *Streptococcus*? From *Pediococcus*?
- 3 How is *Clostridium* distinguished from *Bacillus*? From *Desulfotomaculum*?
- 4 What is the outstanding morphological feature of each of the following? *Caryophanon*. *Arthrobacter*. *Nocardia*. *Sarcina*. *Corynebacterium*.
- 5 Name three genera that contain mycolic acids in their cell walls. Which contain acid-fast organisms?
- 6 How can *Staphylococcus* be distinguished from *Streptococcus*? Give at least two characteristics.
- 7 What is the outstanding biochemical feature of *Propionibacterium*? Of *Cellulomonas*?
- 8 How has cell-wall chemistry been used in the classification of nocardioform bacteria?
- 9 What kinds of bacteria described in this chapter are noted for the following characteristics?
  - (a) Insect pathogenicity
  - (b) Radiation resistance
  - (c) Growth on the surface of cheeses
  - (d) Pathogens of fish
  - (e) Occurrence of metachromatic granules
  - (f) Cellulose degradation
- 10 Most strains of *Streptococcus* can grow in air, but some strains are anaerobic. The genus *Peptostreptococcus* cannot grow in air, and all strains are anaerobic.



obic. How could you differentiate an anaerobic strain of *Streptococcus* from a strain of *Peptostreptococcus*?

- 11 Some strains of *Actinomyces* produce large amounts of lactic acid. All strains of *Lactobacillus* produce large amounts of lactic acid. How could you differentiate a strain of *Lactobacillus* from *Actinomyces*?

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- Sneath, P. H. A. (ed.): *Bergey's Manual of Systematic Bacteriology*, vol. 2, Williams & Wilkins, Baltimore (in press). This international reference work provides detailed descriptions of the genera and species of bacteria discussed in Chap. 14.
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## Chapter 15

# The World of Bacteria III: Bacteria with Unusual Properties

### OUTLINE *Bergey's Manual of Systematic Bacteriology, Volume 3*

#### Anoxygenic Phototrophic Bacteria

Purple Phototrophic Bacteria • Green Phototrophic Bacteria

#### Oxygenic Phototrophic Bacteria

Cyanobacteria (Blue-Green Algae) • Prochlorophytes

#### Gliding, Fruiting Bacteria

#### Gliding, Nonfruiting Bacteria

#### The Sheathed Bacteria

#### Budding and/or Appendaged Bacteria

Prosthecae Budding Bacteria • Prosthecae Nonbudding Bacteria • Nonprosthecae Budding Bacteria • Nonprosthecae Nonbudding Bacteria

#### Chemolithotrophic Bacteria

The Family *NITROBACTERACEAE* • Sulfur- and Sulfur-Compound-Metabolizing Bacteria • The Family *SIDEROCAPSACEAE*

#### Archaeobacteria

Methanogenic Bacteria • Extreme Halophiles • Thermoacidophiles

The organisms in Volume 3 of *Bergey's Manual* have properties that are quite different from those of the bacteria described in Volumes 1 and 2. Some are distinguished by their unusual type of metabolism. For instance, some of the organisms are **phototrophic**, able to use light as an energy source. Others are **chemolithotrophic**, able to obtain energy by oxidizing inorganic compounds such as ammonia, nitrite, hydrogen sulfide, or ferrous iron. Still others are not distinguished by an unusual metabolism but rather by other features such as the occurrence of **gliding motility** rather than motility by the action of flagella, reproduction by **budding** rather than by binary fission, or special morphological structures such as **sheaths**, **prosthecae**, and **stalks**. Most of the organisms are Gram-negative **eubacteria**, but some, such as those that form methane gas, belong to the major bacterial group known as the **archaeobacteria**, which may stain Gram-negative or Gram-positive. In the present chapter we will describe the



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primitive than oxygenic ( $O_2$ -evolving) phototrophic organisms: geological studies have provided evidence that the atmosphere of our planet was anaerobic at the time life began to develop and that oxygen did not appear in appreciable quantities in the atmosphere until oxygenic bacteria evolved (i.e., bacteria having both photosystem I and photosystem II).

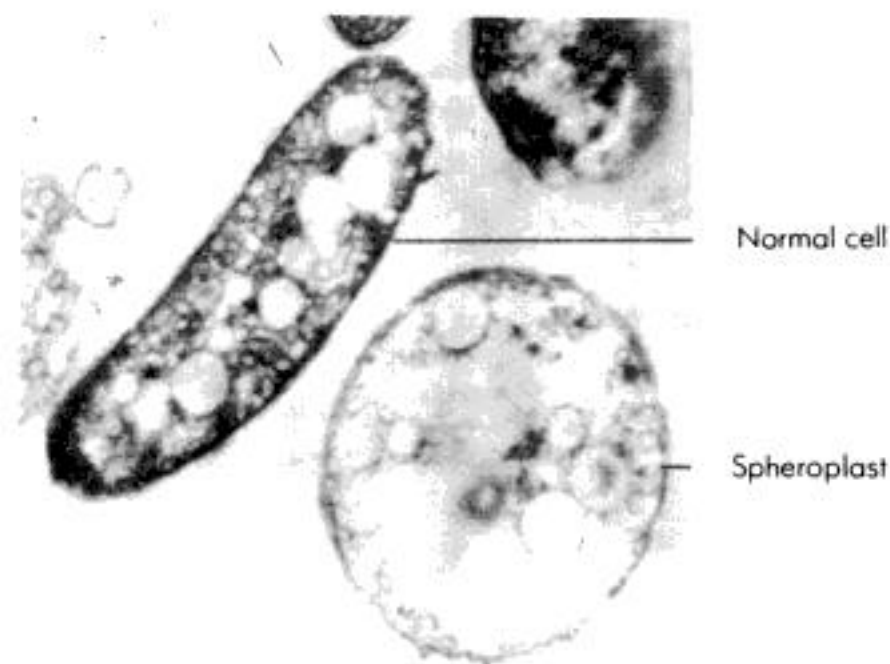
Anoxygenic phototrophic bacteria occur in anaerobic freshwater or marine environments. They may occur beneath the surface of shallow aquatic environments rich in organic matter, such as stagnant ponds, ditches, and salt marsh pools, or, in some instances, they may have a much deeper habitat, as at the bottom of a lake. The bacteriochlorophyll absorbs light most strongly when the light is of long wavelength—about 725 to 745 nm (far red light, at the extreme end of the visible spectrum) to 1035 nm (infrared light, invisible to the human eye). This light is of longer wavelength than that absorbed by the chlorophyll of oxygenic bacteria or oxygenic eucaryotic algae (about 680 nm). Although oxygenic organisms may grow at the surface of a shallow, stagnant pond, they do not absorb far red or infrared light and thus do not prevent it from reaching the anoxygenic phototrophs below. The bacteriochlorophyll and the carotenoid pigments of anoxygenic bacteria can also absorb some light in the blue to blue-green range (between 400 and 550 nm). This becomes important when anoxygenic bacteria occur in the depths of a lake, because blue light can penetrate water to greater distances than red light can.

The color of anoxygenic phototrophic bacteria is determined mainly by the carotenoid pigments rather than by the bacteriochlorophyll, and the anoxygenic phototrophs can be divided into two major groups on the basis of their pigmentation: purple bacteria and green bacteria. Motility, if present in these two groups, is by means of polar flagella, except for the family *Chloroflexaceae* which exhibits a gliding type of motility. Nitrogen can be fixed by purple or green bacteria, but usually only under anaerobic conditions and illumination.

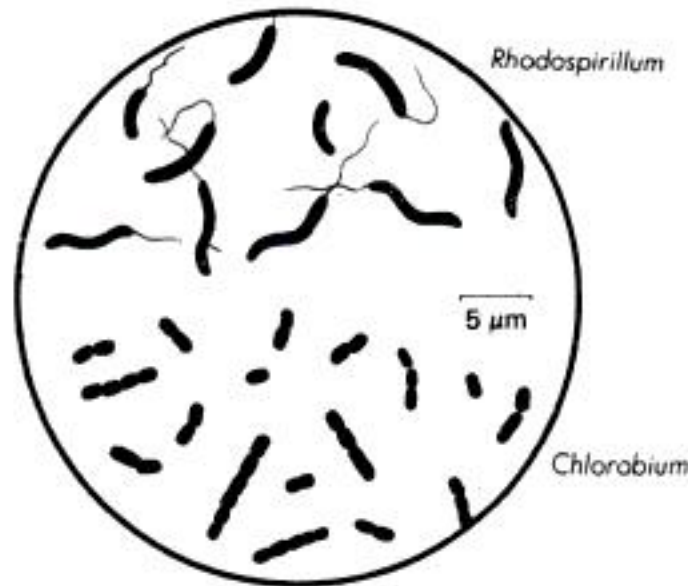
### Purple Phototrophic Bacteria

These organisms contain bacteriochlorophyll types a or b. The pigments that harvest the energy of light (i.e., bacteriochlorophyll and auxiliary carotenoid pigments) are located in the cytoplasmic membrane, which may be greatly

**Figure 15-1.** Electron micrograph of a purple nonsulfur bacterium, *Rhodospirillum rubrum*, showing the small intracellular vesicles that contain the photosynthetic apparatus. The large clear areas are poly- $\beta$ -hydroxybutyrate granules (X14,000). [From E. S. Boatman and H. C. Douglas, *Electron Microscopy*, vol. 2, Fifth International Congress of Electron Microscopy (Philadelphia), Academic, New York, 1962.]

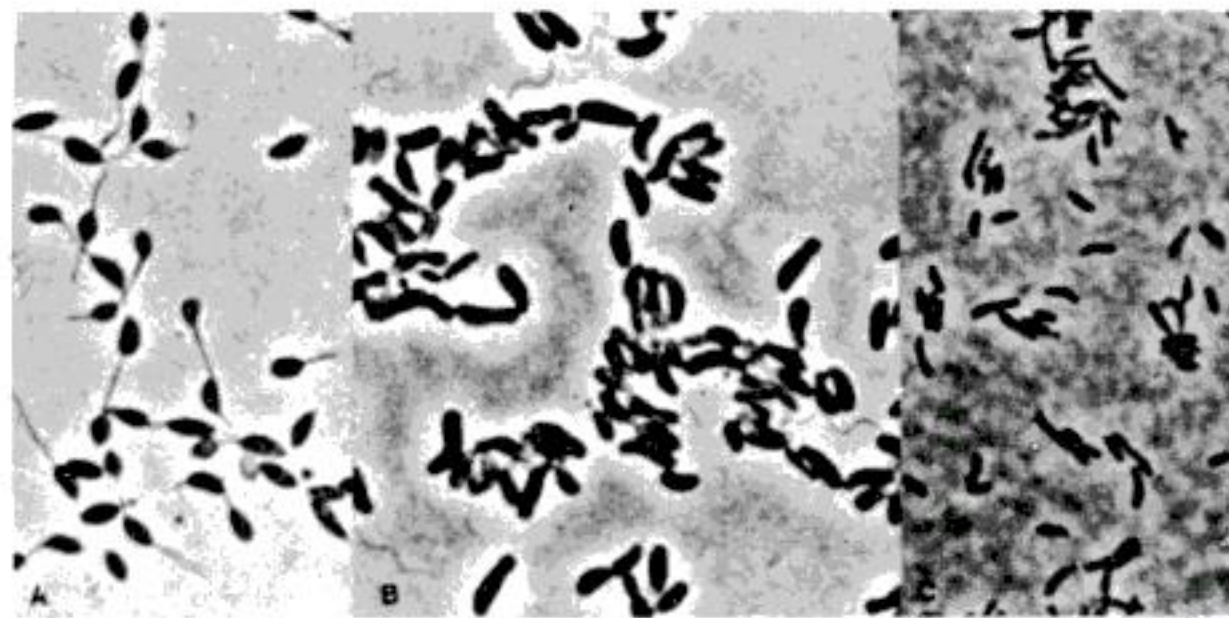






**Figure 15-2.** Drawing of the cells of *Rhodospirillum* (family Rhodospirillaceae) and *Chlorobium* (family Chlorobiaceae). The flagella of *Rhodospirillum* cannot be seen by ordinary staining. (Erwin F. Lessel, illustrator.)

**Figure 15-3.** Some species of the family Rhodospirillaceae. (A) *Rhodomicrobium vannielii*, a prosthecae budding species. The bud forms at the tip of a prostheca and eventually reaches the size of the mother cell. (B) *Rhodopseudomonas acidophila*, a nonprosthecae budding species. The bud is sessile at the pole of the mother cell and separates by constriction when the bud reaches the size of the mother cell. Some bundles of polar flagella can be seen in the field. (C) *Rhodopseudomonas palustris*, a nonprosthecae budding species. The cells are narrower than those in (B). Phase-contrast (X1,464). (From N. Pfennig, *J Bacteriol*, 99:597, 1969.)



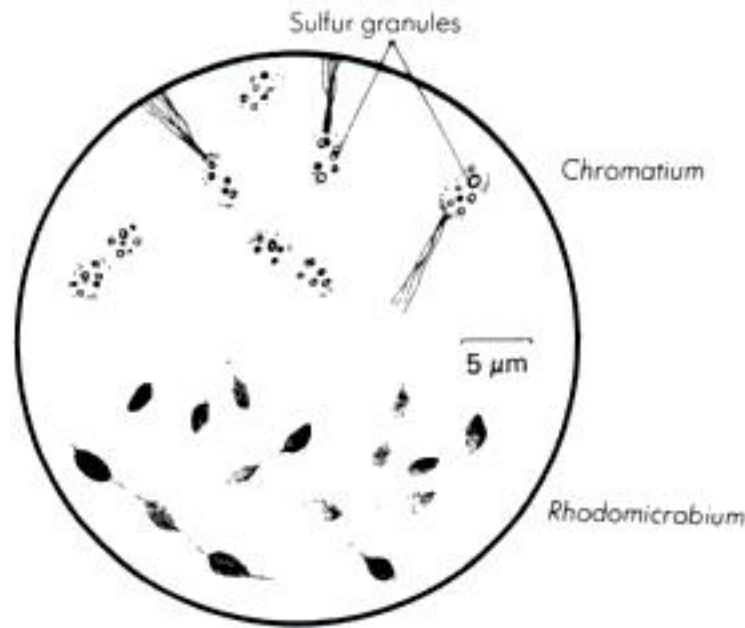
invaginated to form vesicles (see Fig. 15-1), folded layers (lamellae), or tubules. Two families are recognized, as follows.

The family **RHODOSPIRILLACEAE** contains the **purple nonsulfur bacteria**. Cultures appear orange-brown to purple-red under aerobic conditions. Some may be similarly pigmented under anaerobic conditions, but others may be greenish-yellow. The purple nonsulfur bacteria exhibit a diversity of shapes: helical (e.g., *Rhodospirillum*; see Fig. 15-2), nonprosthecae rod-shaped, ovoid, or spherical cells that multiply by binary fission or budding (e.g., *Rhodopseudomonas*; see Figs. 15-3B and C), or ovoid cells that multiply by the formation of buds at the end of prosthecae (e.g., *Rhodomicrobium*; see Figs. 15-3A and 15-4).

The purple nonsulfur bacteria are **photoorganotrophs**: organic substances serve both as carbon sources and as electron donors for the reduction of carbon dioxide. Some species can grow autotrophically by using  $H_2S$  as the electron donor, but only if very low concentrations are provided; none can use elemental sulfur as an electron donor. Photosynthesis occurs only under anaerobic conditions in the light. Some species can also grow under aerobic or microaerophilic conditions in the dark by respiration with organic compounds.

The family **CHROMATIACEAE** contains the **purple sulfur bacteria**. Cultures appear orange-brown to purple-violet. Purple sulfur bacteria may be ovoid to





**Figure 15-4.** Drawing of the cells of *Chromatium* (family Chromatiaceae) and *Rhodospirillum* (family Rhodospirillaceae). The intracellular sulfur granules of *Chromatium* are indicated. (Erwin F. Lessel, illustrator.)

rod-shaped (e.g., *Chromatium*; see Fig. 15-4), coccoid (e.g., *Thiocystis*), or helical (e.g., *Thiospirillum*). Coccal species may be arranged as diplococci (e.g., *Lamprocystis*), in cubical packets (e.g., *Thiosarcina*), or in flat sheets (e.g., *Thiopodia*). Some species contain gas vacuoles.

All genera are capable of photolithotrophic growth, using  $H_2S$  or elemental sulfur as the electron donor for  $CO_2$  fixation. When  $H_2S$  is the electron donor, globules of elemental sulfur are formed, usually within the cells. The sulfur is eventually oxidized to sulfate. Some strains can also grow photoorganotrophically. Most species are anaerobic and cannot grow in the dark even under microaerophilic conditions.

### Green Phototrophic Bacteria

In contrast to the purple bacteria, these organisms contain bacteriochlorophyll types *c* or *d* and minor amounts of *a*. Moreover, cultures are green or brown. The pigments involved in photosynthesis are located in membrane-bound vesicles within the cell; some of these may be attached to the cytoplasmic membrane. Two families are recognized, as follows.

The family **CHLOROBIACEAE** contains the **green sulfur bacteria**. The cells are ovoid, bean-shaped, or rod-shaped (e.g., see the genus *Chlorobium*, Fig. 15-2) and multiply only by binary fission. One genus, *Prosthecochloris*, consists of star-shaped cells, this shape being caused by the production of about 20 prosthecae per cell. Gas vacuoles may occur in some genera.

Green sulfur bacteria live as photolithotrophs, using  $H_2S$  as the electron donor for  $CO_2$  fixation. Granules of elemental sulfur are deposited outside the cells, never within the cells; the sulfur can eventually be oxidized to  $SO_4^{2-}$  (soluble sulfur). The organisms are anaerobic, being incapable of growing in the dark even under microaerophilic conditions.

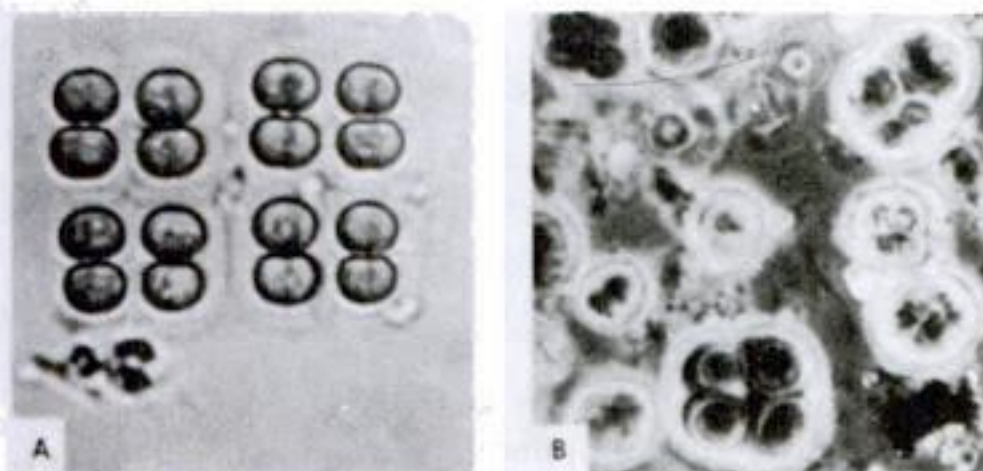
The family **Chloroflexaceae** contains the **green nonsulfur bacteria**. The main genus, *Chloroflexus*, is thermophilic (optimum temperature 52 to 60°C) and occurs in hot springs where it forms green or orange mats. *Chloroflexus* cells occur as filaments or trichomes and exhibit gliding motility. The organisms are mainly photoorganotrophic, as the purple nonsulfur bacteria, but they can also grow as photolithotrophs with  $H_2S$  as the electron donor. In the dark they can grow aerobically as chemoheterotrophs.



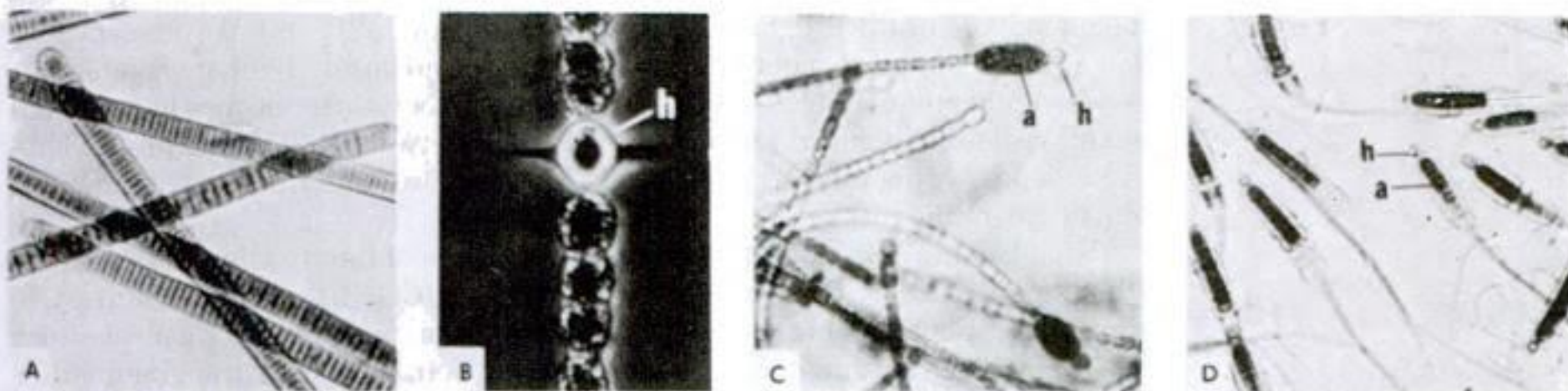
## OXYGENIC PHOTOTROPHIC BACTERIA

### Cyanobacteria (Blue-Green Algae)

These organisms exhibit an enormous diversity of shapes and arrangements, from unicellular cocci (Fig. 15-5) or rods to long trichomes (Fig. 15-6). Gas vacuoles may be formed by many species (Fig. 15-6B). Some cyanobacteria are surrounded by a sheath that surrounds the aggregates (Fig. 15-5B) or trichomes. Unicellular cyanobacteria are usually nonmotile, but trichome-formers generally possess gliding motility. Flagella are absent. Cyanobacteria are widespread in soil, freshwater, and marine habitats. Some are thermophilic, growing in hot springs. Cyanobacteria can grow as mats on the surface of bare soil as primary colonizers. They are important in adding organic matter to the soil and in preventing incipient erosion. Some cyanobacteria grow in symbiosis with other organisms. For example, they may occur as algal symbionts of lichens (see Chap. 18). Some live within the plant bodies of certain liverworts, water ferns, cycads (a class of naked-seed plants), and angiosperms (plants whose seeds are borne within a fruit) where they fix nitrogen. Cyanobacteria have also been associated with certain protozoa, where they are called cyanellae.



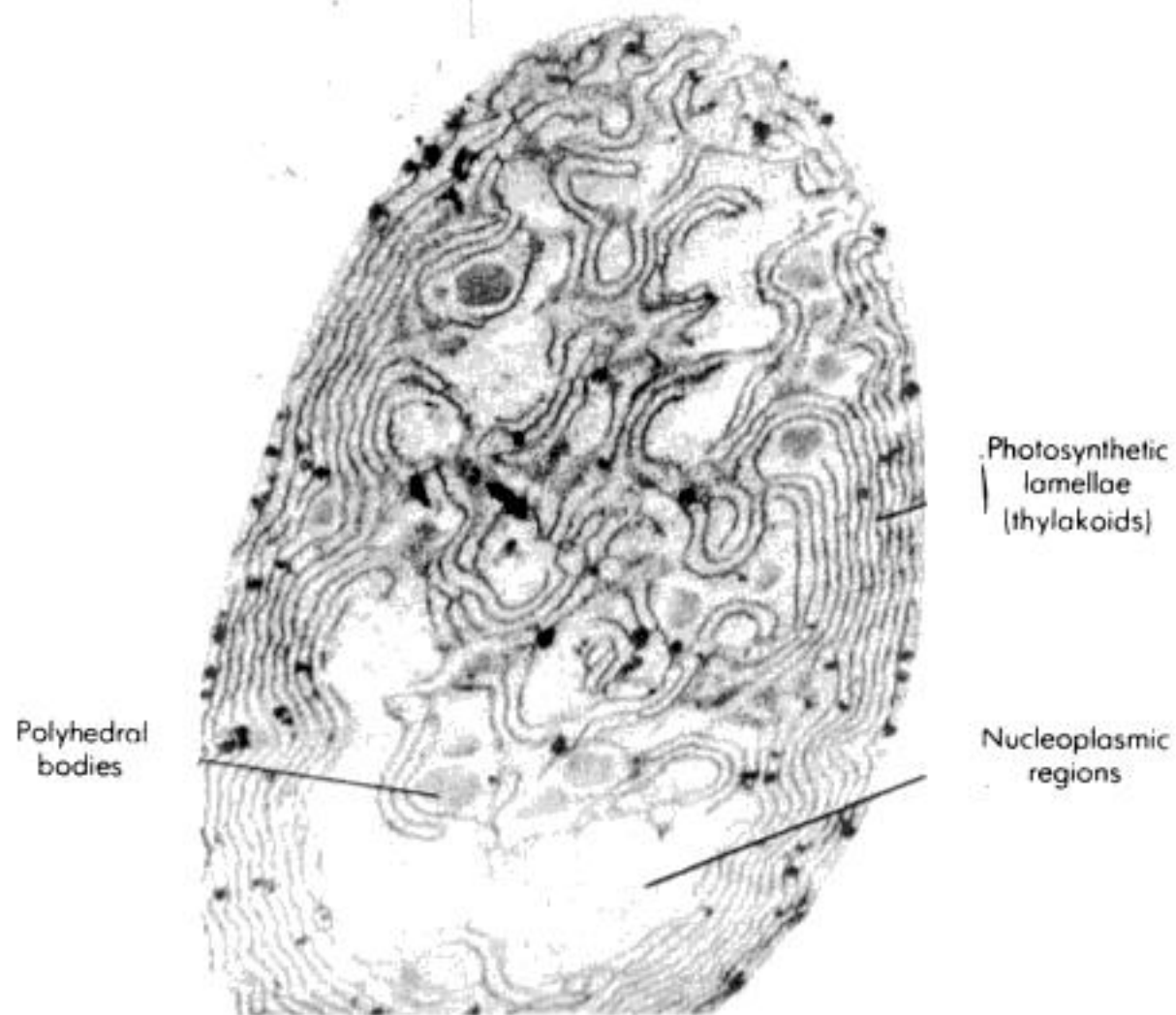
**Figure 15-5.** Examples of coccoid cyanobacteria. (A) *Merismopedia glauca*. The cells are 3 to 5  $\mu\text{m}$  in diameter, and the colony is a flat plate. This species is a common member of the plankton of soft water lakes. (B) *Gloeocapsa rupestris*. The cells are 6 to 9  $\mu\text{m}$  in diameter and are surrounded by a sheath. This species occurs on moist rocks, in soil, or on submerged objects and forms gelatinous masses that are often colored yellow, red, or brown. (Courtesy of George J. Schumacher, State University of New York at Binghamton.)



**Figure 15-6.** Examples of trichome-forming cyanobacteria. (A) *Oscillatoria limosa*. The trichomes consist only of vegetative cells, which are 12 to 18  $\mu\text{m}$  wide. (B) *Anabaena planktonica*. The vegetative cells are 10 to 15  $\mu\text{m}$  wide and contain gas vacuoles (bright areas). A heterocyst (h) is also shown. The heterocysts of this species are unique in that they possess lateral mucilaginous winglike structures, as shown in this photograph. (C) *Cylindrospermum majus*. The vegetative cells are 3 to 5  $\mu\text{m}$  wide and the heterocysts (h), which are always terminal in location, are slightly larger. The akinetes (a) are much larger and are 25 to 30  $\mu\text{m}$  in length. (D) *Gloeotrichia echinulata*. The vegetative cells are 8 to 10  $\mu\text{m}$  wide but decrease in width along the long, tapering trichome. The terminal heterocysts (h) are 8 to 10  $\mu\text{m}$  in diameter, and the akinetes (a) are 10 to 20  $\mu\text{m}$  wide by 45 to 50  $\mu\text{m}$  long. (Courtesy of George J. Schumacher, State University of New York at Binghamton.)



**Figure 15-7.** Section of a vegetative cell of the cyanobacterium *Anabaena azolae*. Most of the photosynthetic lamellae (thylakoids) are peripheral, but some extend into the midportions of the cell. (Courtesy of Norma J. Lang and *J Phycol*, 1:127–134, 1965.)



Cyanobacteria contain chlorophyll *a* rather than bacteriochlorophyll; because of this chlorophyll the cells absorb red light of 680 to 683 nm. Other pigments include water-insoluble carotenoids and also water-soluble *phycobilins*, which are the major light-absorbing pigments in cyanobacteria and which can transmit the energy of absorbed light to the chlorophyll. Blue phycobilins (phycocyanin and allophycocyanin) occur in all cyanobacteria and absorb light at wavelengths between 500 and 650 nm. A red phycobilin, phycoerythrin, occurs in some but not all species and absorbs shorter wavelengths between 470 to 600 nm. Cyanobacteria possessing phycoerythrin have a red or brown color instead of the usual bluish-green hue.

Cyanobacteria are *photolithotrophs*, and because of photosystem II (see Chap. 10) they can use  $H_2O$  as an electron donor for  $CO_2$  fixation, in contrast to anoxygenic phototrophic bacteria. However, some cyanobacteria can also use  $H_2S$  as an electron donor in a manner similar to that used by the green sulfur bacteria. Many cyanobacteria are obligately photolithotrophic, but some can also grow as chemoorganotrophs at a slow rate in the dark.

The photosynthetic apparatus (i.e., chlorophyll *a*, carotenoid pigments, photochemical reaction centers, and the photosynthetic electron transport chain) is contained in the *thylakoids*—flattened membranous sacs located within the cell (see Fig. 15-7). The surface of the thylakoids is studded with granules called *phycobilisomes*, which contain the phycobilin pigments.

Many trichome-forming cyanobacteria can fix  $N_2$ . It seems strange that oxygen-evolving organisms can do this, since nitrogenase is highly oxygen-sensitive; however, in most instances it is not the vegetative cyanobacterial cells that carry





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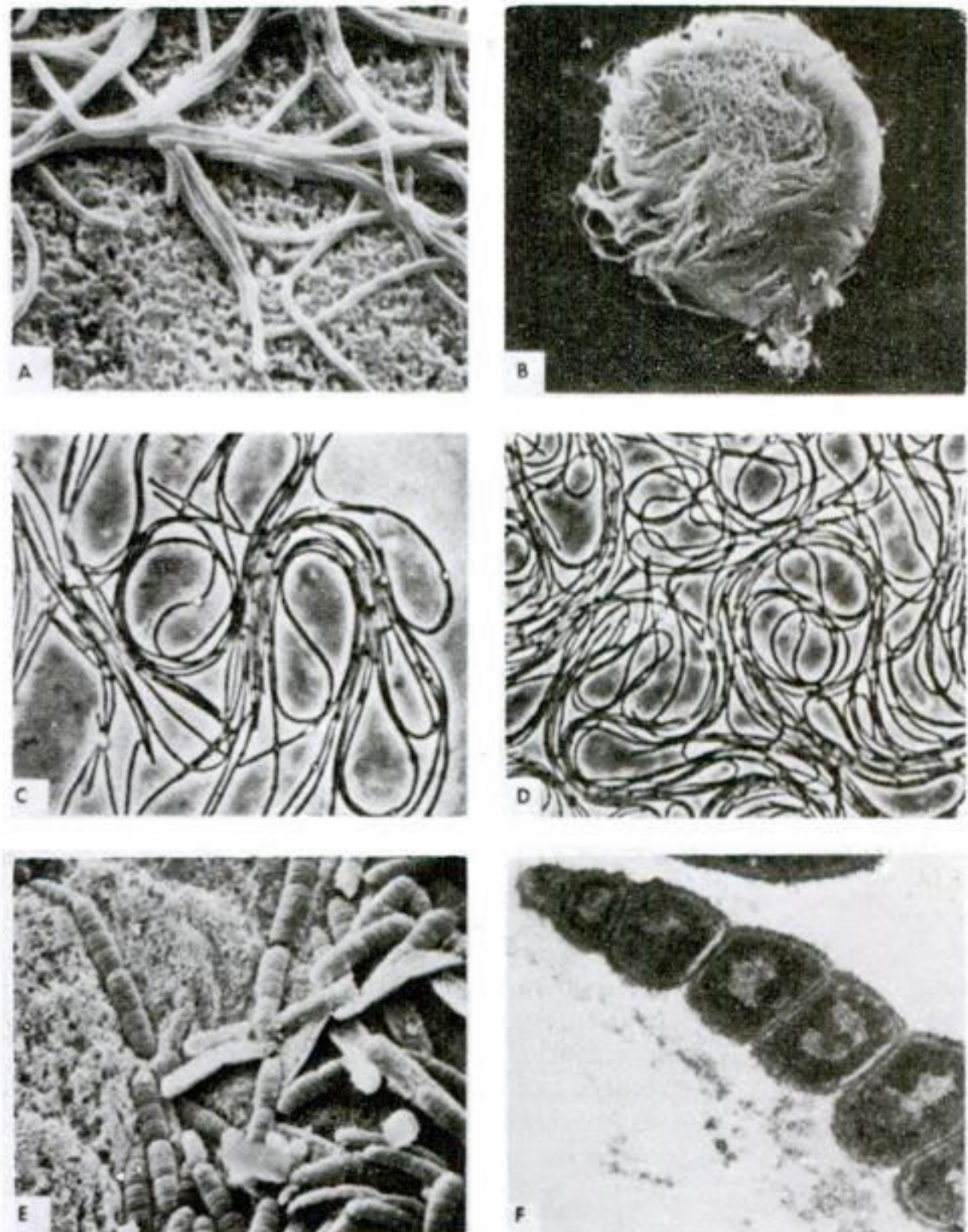
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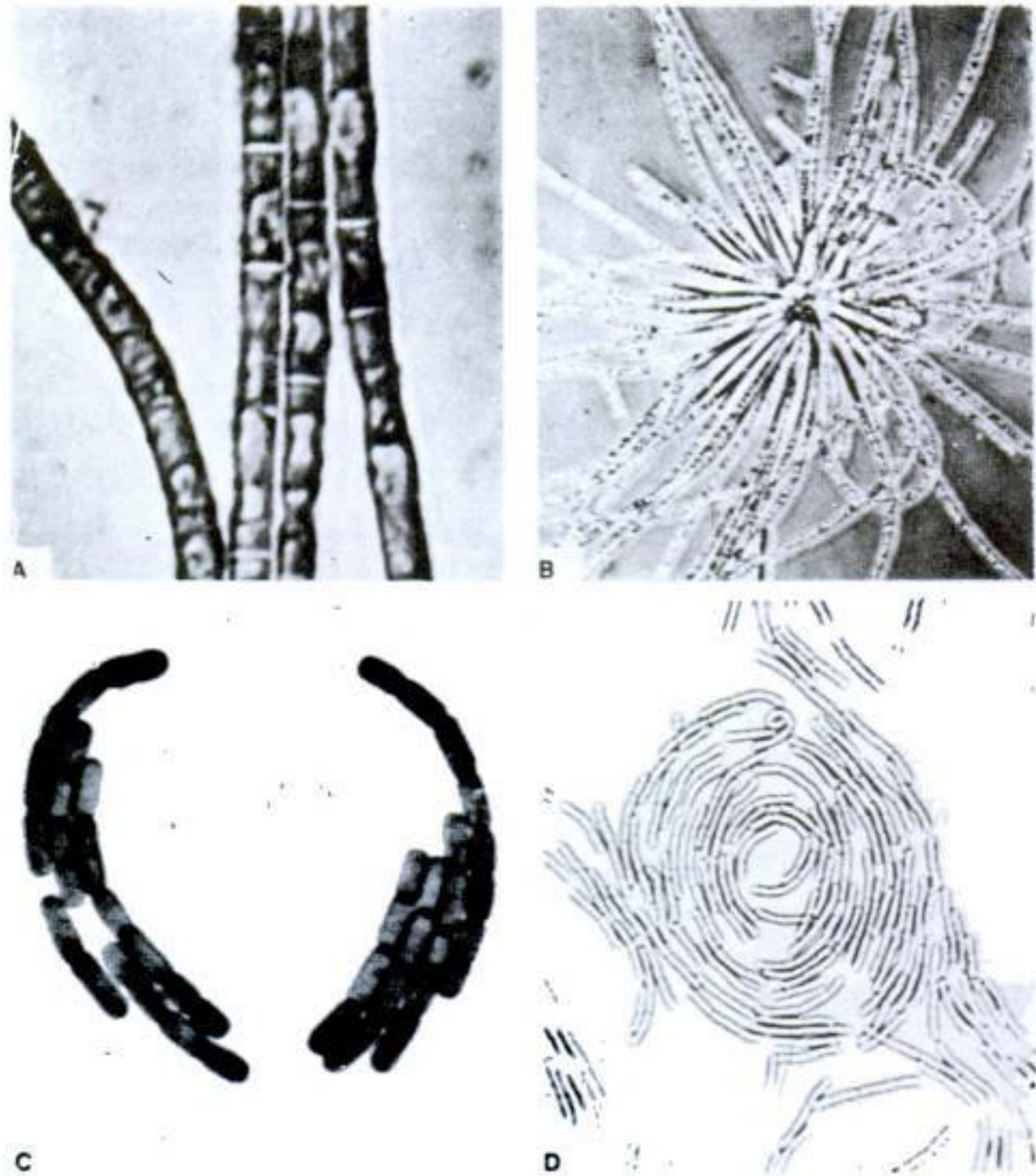
lulose, chitin, pectin, keratin, or even agar. One genus, *Capnocytophaga*, is unusual because of its occurrence in the oral cavity of humans—in the gingival crevice (the space between the surface of the enamel of a tooth and the gum)—and may be involved in periodontal disease. Some aquatic genera such as *Beggiatoa* occur mainly in microaerophilic environments containing  $H_2S$ , which is oxidized by the cells to elemental sulfur; the sulfur accumulates as granules within the cells. Thus, *Beggiatoa* may possibly be an autotrophic organism.

Gliding, nonfruiting bacteria may appear as individual rods or filaments (e.g., *Cytophaga*, *Flexibacter*, or *Vitreoscilla*), or as multicellular trichomes (e.g., *Beggiatoa*, *Simonsiella*, *Saprospira*, or *Thiothrix*). Some of the cells are very long; for example, *Flexibacter* cells may reach 50  $\mu m$  in length (see Fig. 15-10). Some genera such as *Herpetosiphon* and *Flexithrix* produce a sheath which

**Figure 15-10.** Species of gliding, nonfruiting bacteria. (A) *Flexibacter polymorphus*. Cells collected on the surface of a Nucleopore membrane filter (X730). (B) Colony of *F. polymorphus* growing on surface of Nucleopore membrane filter layered over a nutrient agar surface (X100). The holes in the filter are 5.0  $\mu m$  in diameter. (Courtesy of H. F. Ridgeway, Jr., Scripps Institution of Oceanography.) (C) Filaments of the gliding bacterium *Herpetosiphon giganteus* on agar showing “bulbs” (bright spherical enlarged regions) (X500). (D) Same as (C) but at lower magnification (X330). (Courtesy Hans Reichenbach.) (E, F) *Simonsiella* sp. showing cells arranged in apposition to form trichomes with free faces of terminal cells rounded. (E) Scanning electron micrograph (X2,200); (F) transmission electron micrograph of thin section (X20,000). (Courtesy J. Pangborn and Daisy Kuhn.)







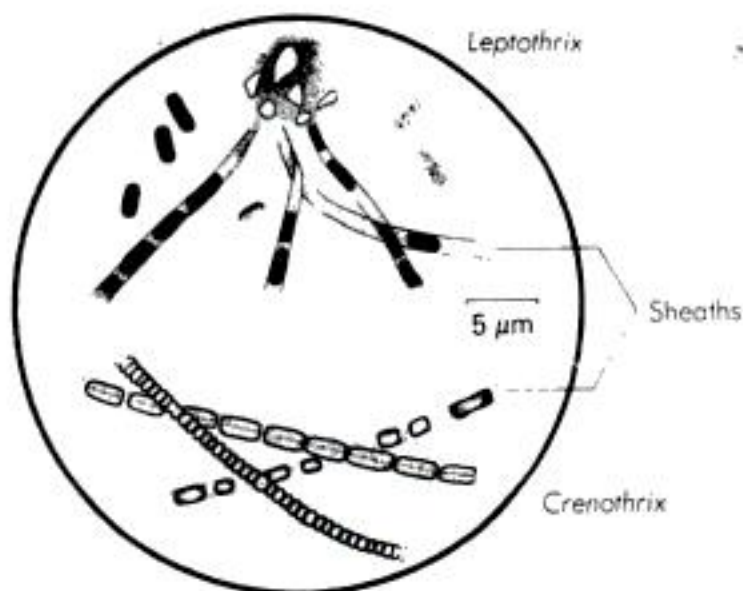
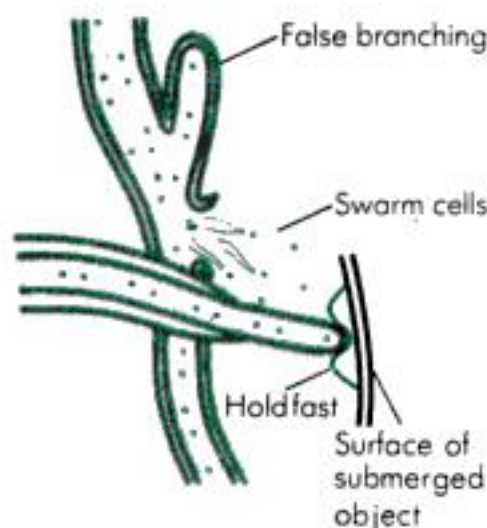
**Figure 15-11.** Gliding, non-fruiting bacteria. (A) Tri-chomes of *Beggiatoa* stained to demonstrate presence of a cell membrane (X2,250). (From H. L. Scoitten and J. L. Stokes, *Arch Mikrobiol*, 42:353, 1962.) (B) Tri-chomes attached to a common object are illustrated in this photomicrograph of *Thiiothrix* sp.. (X420). (Courtesy of F. E. Palmer and E. J. Ordal.) (C) *Vitreoscilla* cell morphology. (Courtesy of G. J. Hageage, Jr.) (D) Pattern of cell arrangement of *Vitreoscilla*. (Courtesy of V. B. D. Skerman.)

encloses the individual cells. Examples of the morphological features characteristic of gliding, nonfruiting bacteria are shown in Figs. 15-10 and 15-11 (see also Fig. 5-4).

## THE SHEATHED BACTERIA

Bacteria in this group are Gram-negative, aerobic, and nonphototrophic, and are characterized by the formation of a sheath surrounding a chain of cells or a trichome. The sheaths of some genera are encrusted with ferric and manganic oxides. Sheathed bacteria inhabit freshwater and marine environments. Among the genera included in the group are: *Sphaerotilus*, *Leptothrix* (Fig. 15-12), *Haliscomenobacter*, *Streptothrix*, *Lieskeella*, *Phragmidiothrix*, *Crenothrix* (Fig. 15-12), and *Clonothrix*. Only the first three genera have been isolated; the others are characterized solely on the basis of their distinctive morphology as observed in samples from natural sources. One of the cultivatable genera, *Sphaerotilus*, is discussed here.





**Figure 15-12.** Drawing of sheathed bacteria of the genus *Leptothrix* and *Crenothrix*. (Erwin F. Lessel, illustrator.)

**Figure 15-13.** Sheathed bacteria. Drawing of *Sphaerotilus* showing sheath, false branching, and motile swarmers. When a swarmer encounters a solid object, it can become attached by a holdfast, formed by secreting a sticky substance that hardens. Sheath formation begins at the holdfast. (Redrawn from K. A. Bisset, *Bacteria*, E. and S. Livingstone, Ltd., Edinburgh, 1952.)

## *Sphaerotilus*

The cells typically occur as chains of rods enclosed within a sheath, as shown in Fig. 5-17. The sheath may branch, giving the impression that the cells are branched; however, this is recognized as “false branching” (Fig. 15-13). The cells that emerge from an open end or a break in the sheath are called *swarm cells*. The hollow sheaths accumulate. The swarm cells are rod-shaped and possess polar or subpolar flagella; they thus resemble pseudomonads in appearance. *S. natans* is a common species that normally occurs in polluted waters, and its sheaths, of organic composition, are thin and colorless. In unpolluted water containing iron, iron hydroxide may be deposited in or on the sheaths, which turn yellow-brown and may become encrusted with ferric iron. Hence, these organisms are sometimes referred to as “iron bacteria.”

## BUDDING AND/OR APPENDAGED BACTERIA

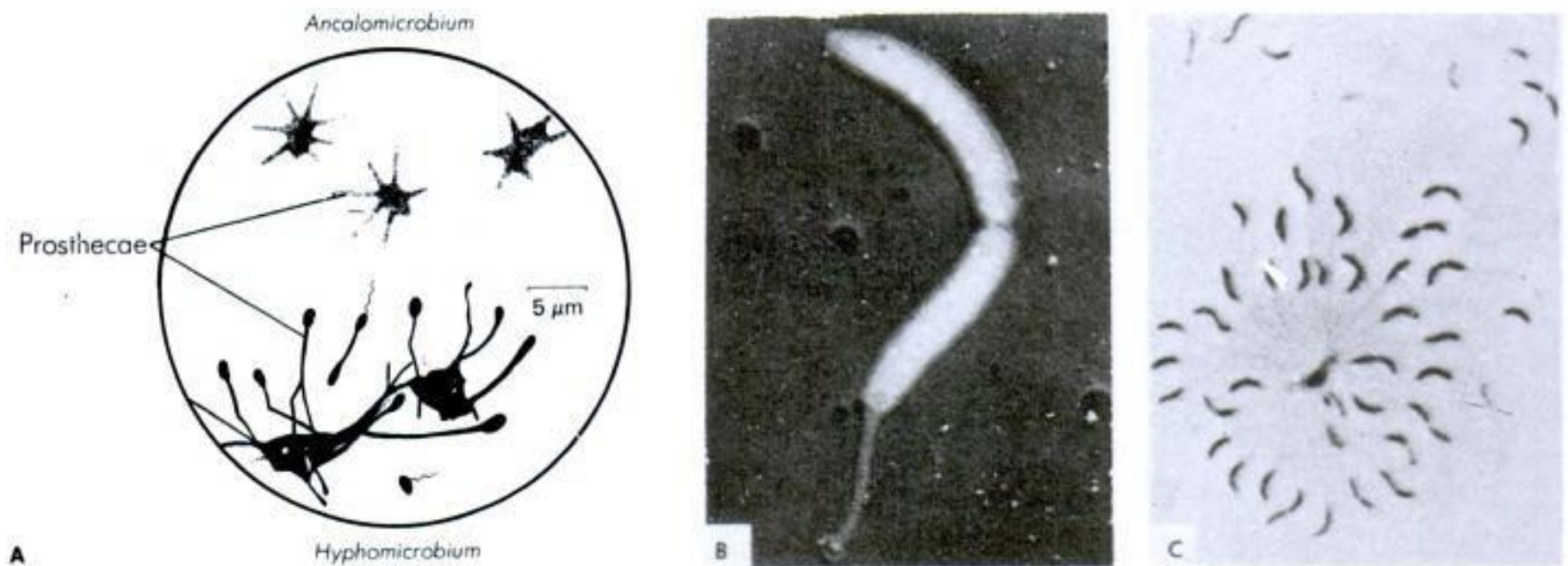
This group of nonphototrophic Gram-negative bacteria is characterized by the formation of *prosthecae* (extensions of the cell wall and cytoplasmic membrane) or *stalks* (nonliving ribbonlike or tubular appendages that are excreted by the cell) and/or by the asymmetric mode of reproduction called budding. The organisms range from aerobic to microaerophilic to facultatively anaerobic. Although nonphototrophic, some genera have morphologically similar counterparts in the phototrophic group of bacteria. A few examples of budding and/or appendaged bacteria are described below.

### Prosthecae Budding Bacteria

#### *Hyphomicrobium*

Individual cells are initially coccoid and flagellated but mature into oval or bean-shaped cells. Prosthecae are produced at either one or both ends of a cell. Buds develop at the tips of the prosthecae (see Fig. 15-14A and also Fig. 7-1E), and as they mature they separate from the prosthecae. Hyphomicrobia are aerobic and chemoorganotrophic; however, their morphology is similar in many ways to that of the phototrophic genus *Rhodospirillum rubrum* (see Fig. 15-3). Hy-





**Figure 15-14.** Budding and/or appendaged bacteria. (A) Drawing of cells of *Hyphomicrobium* and *Ancalomicrobium*. (Erwin F. Lessel, illustrator.) (B) *Caulobacter*, undergoing binary fission. The upper cell possesses a polar flagellum; the lower cell has a prostheca with terminal holdfast (X13,000). (Courtesy of A. L. Houwink and W. van Iterson, *Biochem Biophys Acta*, 5:10, 1950.) (C) *Caulobacter* cells attached to a common holdfast and exhibiting a rosette pattern. (Courtesy of V. B. D. Skerman.)

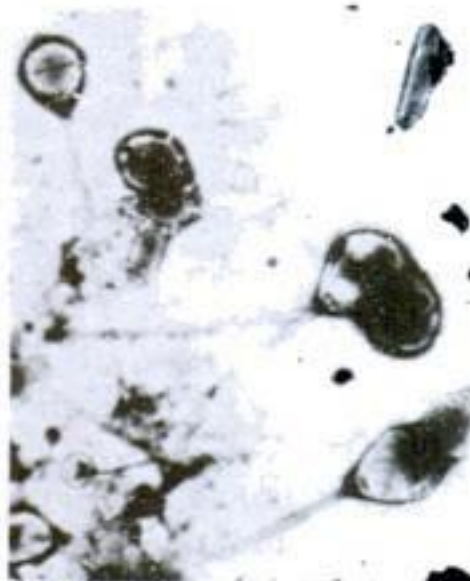
phomicrobia have been found widely in soils of all continents, as well as in numerous aquatic environments.

### *Ancalomicrobium*

These facultatively anaerobic aquatic bacteria have three to eight, long, tapering prosthecae per cell (see Fig. 15-14A and also Fig. 5-18). Buds are formed directly from one position on the mother cell, never from the prosthecae. A genus of phototrophic green sulfur bacteria, *Ancalochloris*, bears a morphological resemblance to *Ancalomicrobium*.

### **Prosthecae Nonbudding Bacteria**

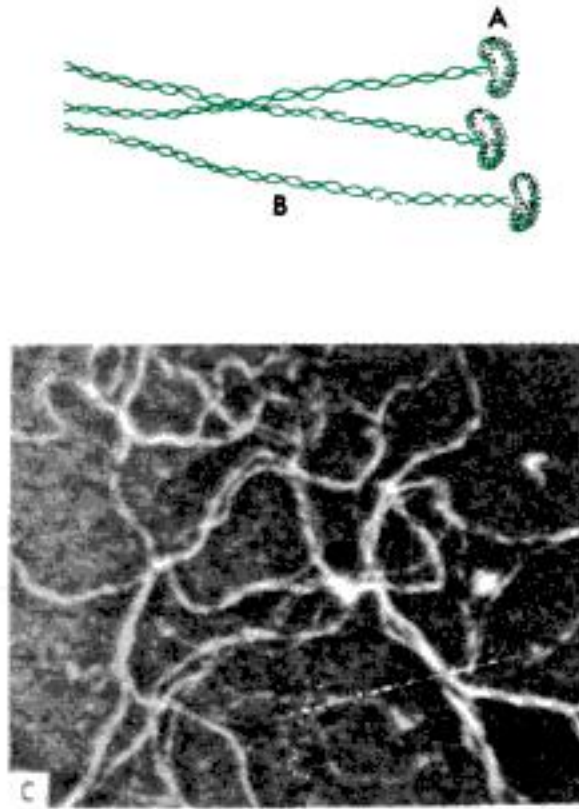
#### *Caulobacter*



In this genus of aerobic organisms, an individual cell is either a straight or curved rod with a single prostheca. The daughter cell arises by binary fission; it possesses a single polar flagellum (Fig. 15-14B) and is termed a swarm cell. The free motile swarm cell secretes an adhesive material (holdfast) at the end of the cell where the flagellum is located; eventually, a prostheca is produced at the same pole and the holdfast becomes located at the end of the prostheca. The flagellum is usually lost during formation of the prostheca. By means of the holdfast, cells may become attached to each other to form rosettes (Fig. 15-14C) or they may become attached to some other substance. *Caulobacters* are normally found in salt water and freshwater and have the ability to grow in environments with very low concentrations of nutrients.

**Figure 15-15.** Electron micrograph of stalked cells of a member of the *Blastocaulis-Planctomyces* group, morphotype II. A bud developing from a mother cell can be seen at the lower right (X4,400). (Courtesy of Jean M. Schmidt, Arizona State University.)





**Figure 15-16.** *Gallionella*, showing the kidney-shaped cells (A) bearing twisted stalks (B). (C) *Gallionella* sp. from salt water showing long entangled stalks (dark-phase microscopic preparation; X147). (From J. M. Sharpley, *Appl Petrol Microbiol*, 9:380, 1961.)

### Nonprosthecate Budding Bacteria

Bacteria of the *Blastocaulis-Planctomyces* group occur in freshwater, brackish, or marine environments. The cells are spherical or ovoid and possess a stalk with a holdfast at the distal end. Budding occurs directly from a mother cell, as shown in Fig. 15-15.

### Nonprosthecate, Nonbudding Bacteria

#### *Gallionella*

A twisted ribbonlike stalk extends from the middle of the curved or kidney-shaped cells (Fig. 15-16). The cells reproduce by binary fission. The organisms grow under microaerophilic conditions where both  $O_2$  and a supply of ferrous iron are being continuously supplied. Since ferrous iron spontaneously oxidizes in the presence of  $O_2$ , these environmental conditions are unusual but can be met in aerated, iron-removal water-treatment plants, drainage from certain coal mines, or in various thermal springs. It is likely that *Gallionella* is autotrophic, obtaining energy by oxidizing ferrous iron to the ferric form. Because of its ability to form insoluble oxidized iron compounds, *Gallionella* may cause problems, such as clogging, in pipelines of water systems.

### CHEMOLITHOTROPHIC BACTERIA

Three distinct metabolic types constitute this category of Gram-negative autotrophic bacteria, namely:

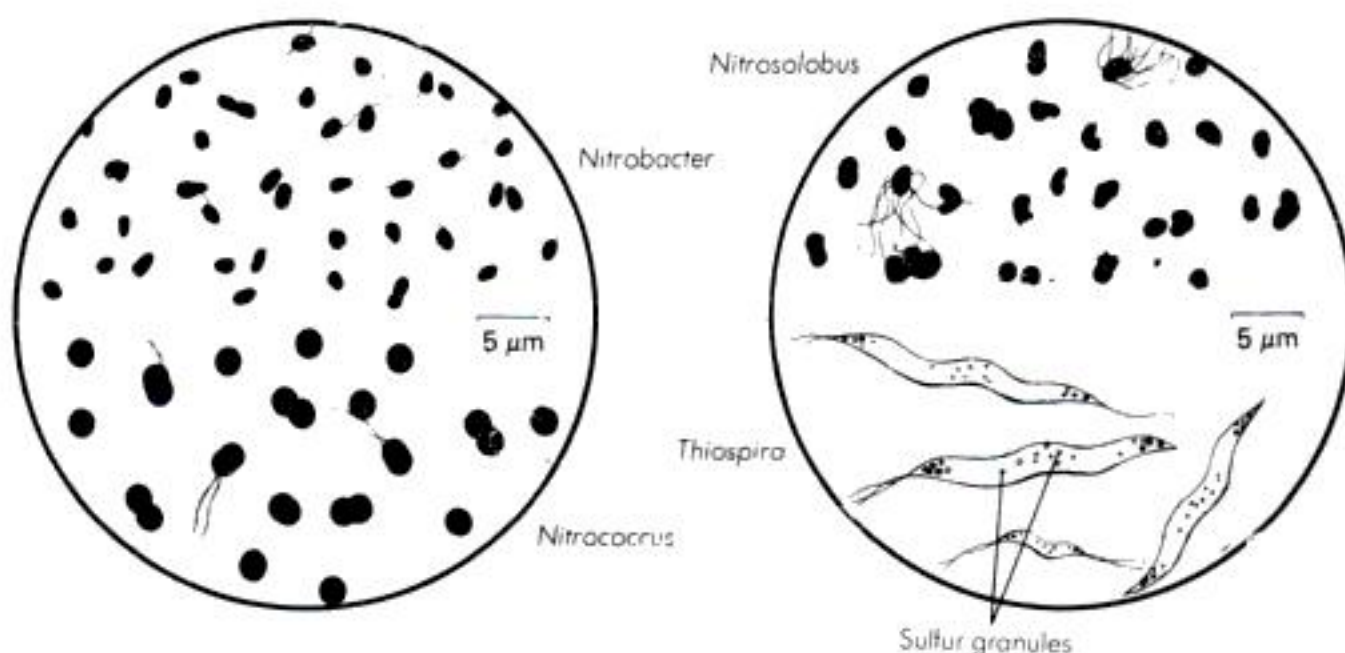
- 1 Obtain energy by oxidizing ammonia or nitrite (family *Nitrobacteraceae*)
- 2 Obtain energy by oxidizing sulfur or sulfur compounds; not assigned to any family
- 3 Deposit iron and/or manganese oxides (family *Siderocapsaceae*)

### The Family *NITROBACTERACEAE*

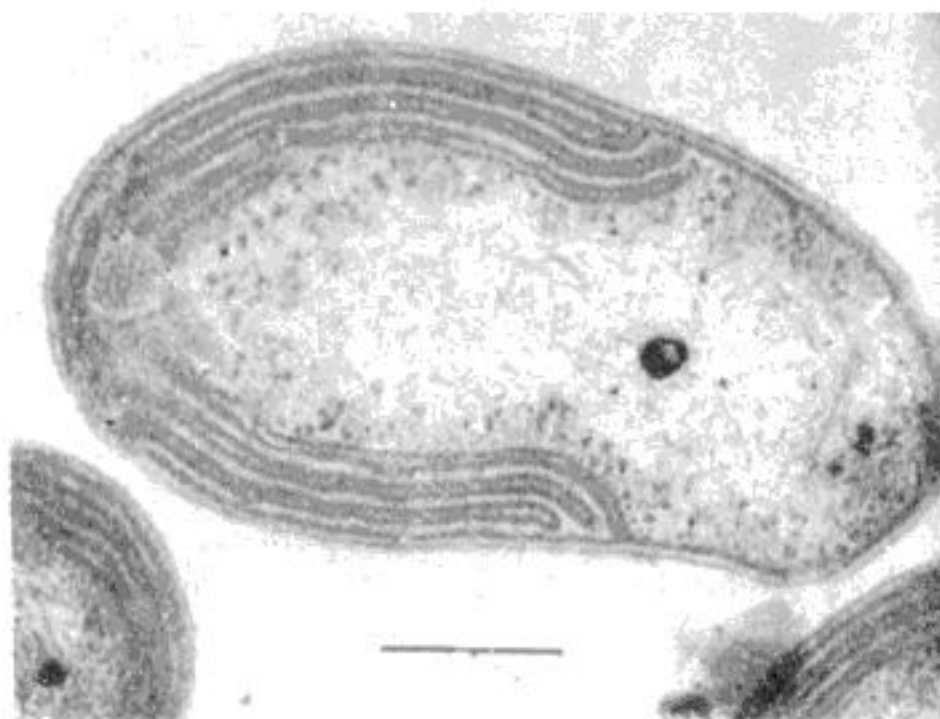
The “nitrifying bacteria,” as these organisms are called, include species of diverse morphological types—rods, cocci, and helical cells. They are nonmotile or motile by subpolar or peritrichous flagella. They are aerobic autotrophs, incapable of chemoheterotrophic growth with the exception of one species, *Nitrobacter winogradskyi*. The nitrifying bacteria comprise two distinct metabolic groups in terms of reactions that provide energy: (1) those which oxidize



**Figure 15-17.** Drawing of chemolithoautotrophic bacteria. Nitrifying bacteria of the genera *Nitrobacter*, *Nitrococcus*, and *Nitrosolobus*, and sulfide-oxidizing bacteria of the genus *Thiospira*. (Erwin F. Lessel, illustrator.)



**Figure 15-18.** Electron micrograph of a longitudinal section of *Nitrobacter winogradskyi* showing intracellular membranes derived from invagination of the cytoplasmic membrane. This species reproduces by budding. The bar indicates 0.25 μm. (From S. W. Watson, *Int J Syst Bacteriol*, 21:254–270, 1971.)



nitrite to nitrate, the generic names beginning with the prefix nitro, e.g., *Nitrobacter* (Fig. 15-17), *Nitrococcus* (Fig. 15-17), and *Nitrospina*; and (2) those which oxidize ammonia to nitrite, the generic names beginning with the prefix nitroso, e.g., *Nitrosolobus* (Fig. 15-17), *Nitrosomonas*, *Nitrosovibrio*, *Nitrosococcus*, and *Nitrospira*. Some species have extensive invaginations of the cytoplasmic membrane [e.g., see *N. winogradskyi* (Fig. 15-18) and *Nitrosococcus oceanus* (Fig. 5-27)]. Nitrifying bacteria are commonly found in soils, where they play an important role in the nitrogen cycle and in maintaining the fertility of soil. They are discussed in more detail in Chap. 25.

### Sulfur- and Sulfur-Compound-Metabolizing Bacteria

The bacteria of this group can be divided into those genera which have been isolated into pure culture and those which have not yet been isolated (noncultivable). The cultivatable genera contain Gram-negative short rods (*Thiobacillus*) or helical cells (*Thiomicrospira*); most species are motile by means of polar flagella. Both genera are widely distributed in soil, freshwaters, coal-mine



drainage waters, and marine environments. They derive energy from the oxidation of reduced sulfur compounds, including sulfides, elemental sulfur, thiosulfate, polythionates, and sulfite. The final oxidation product is sulfate. One species, *Thiobacillus ferrooxidans*, can also derive energy by oxidizing ferrous iron to the ferric form. Some species are obligate autotrophs (e.g., *Thiobacillus thioparus*, *Thiobacillus thiooxidans*, and *Thiomicrospira pelophila*), and others are facultative autotrophs (e.g., *Thiobacillus novellus*). Most species are strictly aerobic, but a few can also grow anaerobically with nitrate as the terminal electron acceptor (e.g., *Thiobacillus denitrificans* and *Thiomicrospira denitrificans*). Some species are acidophilic. For instance, *T. thiooxidans* grows best at pH values of 2 to 5; moreover, it produces so much sulfuric acid that the pH of the medium may decrease to 0 or lower.

The noncultivable genera include *Thiobacterium*, *Macromonas*, *Thiovulum*, *Achromatium*, and *Thiospira*. These genera are recognized by their distinctive morphological features as observed in samples from natural sources. Because these organisms have not been isolated, their metabolic nature cannot yet be described with certainty; however, they are probably chemolithotrophic because (1) they occur only in environments having a continuous supply of  $O_2$  and  $H_2S$ , and (2) they accumulate sulfur granules intracellularly (e.g., see *Thiospira*, Fig. 15-17).  $H_2S$  is unstable (oxidized nonbiologically) in the presence of  $O_2$ ; consequently, the environments that support growth are those located between the aerobic and anaerobic zones of freshwater or marine environments or in  $H_2S$ -bearing springs.

### The Family SIDEROCAPSACEAE

This group includes unicellular, aerobic to microaerophilic organisms which do not form prosthecae or stalks and which deposit iron or manganese oxides on or in capsules or exocellular slime. The group includes such genera as *Siderocapsa*, *Siderococcus*, *Siderocystis*, and *Naumanniella*. (The prefix *sidero* which occurs in some of these names is derived from the Greek noun *siderus* meaning "iron.") There is some question as to whether they are indeed chemolithotrophs, since they generally occur in environments containing not only iron and manganese but also organic matter (stagnant waters, swamp ditches, mud, etc.). Only three species, *Siderocapsa eusphaera*, *Siderocapsa geminata*, and *Naumanniella polymorpha* have apparently been isolated (although their morphology differs from that seen in nature), but only *N. polymorpha* seems to be capable of autotrophic growth. The other species have not been isolated and are recognized solely by their morphological features as observed in samples from natural sources.

## ARCHAEOBACTERIA

Evidence based on studies of ribosomal RNA indicates that archaeobacteria and eubacteria diverged at a very early stage in the evolution of life on earth (see Chap. 3). The phylogenetic gap that exists between the two groups is reflected by certain phenotypic differences, some of which are summarized in Table 15-2. One should recognize, however, that archaeobacteria do not comprise a homogeneous group. Just as great heterogeneity occurs among the eubacteria, so do the various kinds of archaeobacteria differ from each other in terms of morphology, chemical composition, metabolism, and habitat. At present, three



**Table 15-2.** Some Differences between Archaeobacteria and Eubacteria

Characteristic	Archaeobacteria	Eubacteria
Cell Walls		
Peptidoglycan containing muramic acid and D-amino acids is present	—	+
Lipids of Cytoplasmic Membrane		
Long-chain fatty acids bound to glycerol by ester linkages	—	+
Long-chain branched alcohols (phytanols) bound to glycerol by ether linkages	+	—
Properties Related to Protein Synthesis		
First amino acid to initiate a new polypeptide chain is		
Methionine	+	—
N-Formylmethionine	—	+
Translation process sensitive to action of		
Diphtheria toxin*	+	—
Chloramphenicol†	—	+

\* For the action of diphtheria toxin, see Chap. 31.

† For the action of the antibiotic chloramphenicol, see Chap. 24.

main categories of archaeobacteria are recognized: the methane-producers (methanogens), the red extreme halophiles, and the thermoacidophiles.

### Methanogenic Bacteria

These archaeobacteria are stringent anaerobes that share an ability to obtain energy for growth by oxidizing compounds such as  $H_2$  or formate, and utilizing the electrons thus generated to reduce  $CO_2$  with the formation of methane gas ( $CH_4$ ). Some genera can grow as autotrophs, using  $H_2$  and  $CO_2$  as sole sources of carbon and energy; others require additional substances such as vitamins, acetate, amino acids, or organic sulfur compounds. Most species grow better in complex media (e.g., containing yeast extract) than in inorganic media.

At least two unusual coenzymes occur in almost all methanogens that have not been found in other bacteria: Coenzyme M, involved in methyl transfer reactions, and Coenzyme  $F_{420}$ , a flavin-like compound involved in the anaerobic electron transport system of these bacteria. The latter coenzyme fluoresces under ultraviolet light. Its presence can be detected by observing the organisms with a fluorescence microscope; this provides a convenient means to identify methanogens.

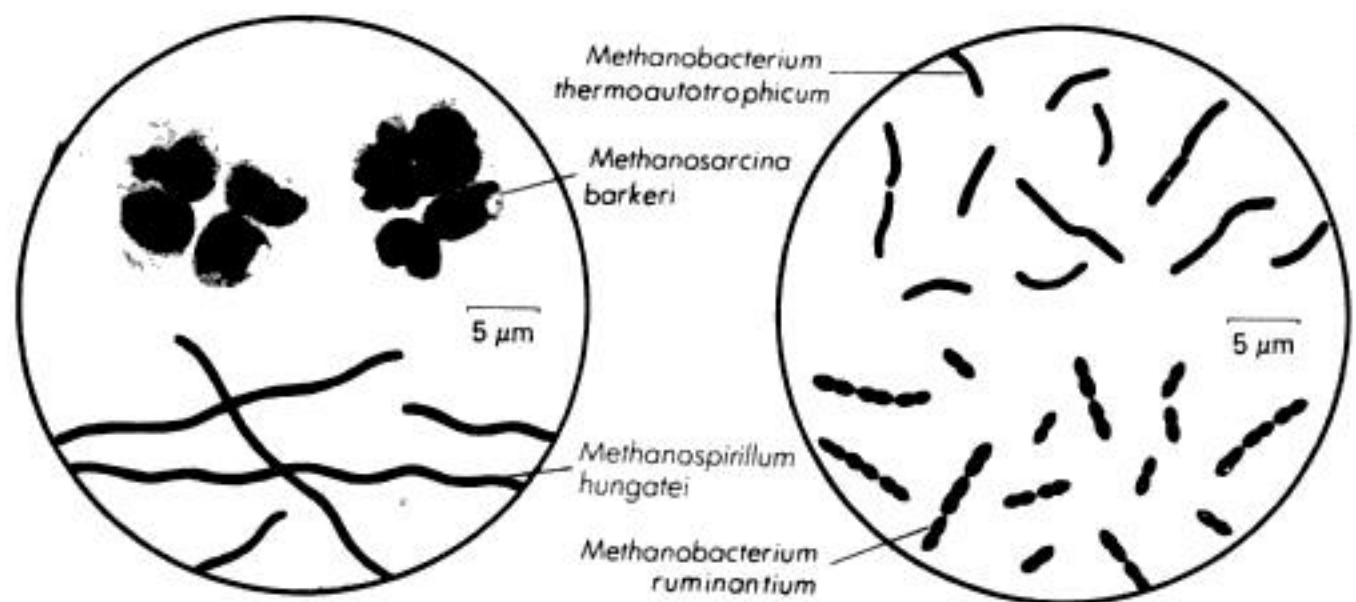
The genera of methane-producing bacteria are differentiated on the basis of morphology and Gram reaction (see Table 15-3 and Fig. 15-19). Differences in cell wall composition have been found to correlate with these genera (Table 15-3). The cell walls of two genera consist of pseudomurein, which differs from eubacterial peptidoglycan by (a) substitution of N-acetyltalosaminuronic acid for N-acetylmuramic acid, and (b) by a tetrapeptide composed entirely of L-amino acids, with glutamic acid at the C-terminal end.

Methanogens occur in various anaerobic habitats rich in organic matter which nonmethanogenic bacteria ferment to produce  $H_2$  and  $CO_2$ . Such habitats include marshes, swamps, pond and lake mud, marine sediments, the intestinal tract of humans and animals, the rumen of cattle, and anaerobic sludge digesters in sewage-treatment plants.



**Table 15-3.** Methanogenic Bacteria

Genus	Morphology	Motility	Wall Composition
<i>Methanobacterium</i>	Gram-positive to Gram-variable long rods	—	Pseudomurein
<i>Methanobrevibacter</i>	Gram-positive lancet-shaped cocci or short rods	—	Pseudomurein
<i>Methanomicrobium</i>	Gram-negative short rods	+, single polar flagellum	Protein
<i>Methanogenium</i>	Gram-negative pleomorphic cocci	+, peritrichous flagella	Protein
<i>Methanospirillum</i>	Gram-negative curved rods or long wavy filaments	+, polar flagella	Protein; an external sheath is present
<i>Methanosarcina</i>	Gram-positive cocci in clusters	—	Heteropolysaccharide
<i>Methanococcus</i>	Gram-negative pleomorphic cocci	+, one flagellar tuft	Protein with trace of glucosamine

**Figure 15-19.** Drawing of the cells of various methane-producing bacteria. (Erwin F. Lessel, illustrator.)

### Extreme Halophiles

These chemoorganotrophic, aerobic bacteria require approximately 17 to 23 percent NaCl for good growth. They stain Gram-negative and range from rod- or disk-shaped cells (the genus *Halobacterium*) to cocci (the genus *Halococcus*). They occur in salt lakes (e.g., the Dead Sea and the Great Salt Lake), industrial plants that produce salt by solar evaporation of sea water, and salted proteinaceous materials such as salted fish (in which they may cause spoilage). The colonies are a red to orange color due to carotenoids which seem to protect the cells against the damaging effect of sunlight.

At high NaCl concentrations the cells resist dehydration by maintaining a high intracellular osmotic concentration of KCl. The cytoplasmic membrane and ribosomes are stable only at high concentrations of KCl, and the enzymes are active only at high levels of either KCl or NaCl. *Halobacterium* cell walls are



composed of protein subunits that are held together only in the presence of salt; thus if the level of NaCl falls below about 10 percent, the cells lyse. On the other hand, the walls of *Halococcus* cells are composed of a complex heteropolysaccharide that is stable even at low salt concentrations.

### ATP Synthesis

Halobacteria are mainly aerobic. As in other aerobic organisms, an electron transport chain generates a protonmotive force which in turn drives ATP synthesis (see Chap. 10). Halobacteria can also generate ATP by fermenting the amino acid arginine; this allows them to grow anaerobically. However, a third method of ATP generation is unique to halobacteria. At low oxygen levels, patches of a purple pigment called *bacteriorhodopsin* are formed in the cell membrane. (This pigment is so named because of its similarity to the photosensitive pigment rhodopsin that occurs in the retinal rods of higher vertebrates.) When cells containing the bacteriorhodopsin are exposed to light, the pigment bleaches. During this bleaching, protons (hydrogen ions,  $H^+$ ) are extruded to the outside of the membrane, thus creating a protonmotive force which in turn drives ATP synthesis. Thus, like the phototrophic bacteria considered earlier in this chapter, halobacteria possess a mechanism for light-driven synthesis of ATP; however, they possess no bacteriochlorophyll.

### Thermoacidophiles

These aerobic Gram-negative archaeobacteria are characterized by a remarkable ability to grow under highly acidic conditions at high temperatures. Two genera included in this group are described below.

#### *Thermoplasma*

These chemoorganotrophic organisms resemble mycoplasmas (see Chap. 13) by lacking a cell wall and forming tiny "fried-egg" colonies. Like mycoplasmas, the cells are pleomorphic, ranging from spherical to filamentous. The optimum temperature for growth is 55 to 59°C (maximum, 62°C; minimum, 40°C), and the optimum pH is 2 (maximum, 4; minimum, 1). Cells undergo lysis at a neutral pH. Thermoplasmas have been isolated from piles of burning coal refuse.

#### *Sulfolobus*

Cells of this genus are spherical or lobe-shaped. Unlike thermoplasmas, a cell wall is present (composed mainly of protein). Various species have temperature optima ranging from 70 to 87°C. The optimum pH is 2 (maximum, 4; minimum, 1). *Sulfolobus* species are facultatively autotrophic. They can grow as chemolithotrophs when supplied with elemental sulfur as an electron donor. Alternatively, they can grow as chemoorganotrophs in media containing organic substrates. In nature, the organisms are predominant in acidic hot springs.

## QUESTIONS

- 1 List the major differences between the families of anoxygenic phototrophic bacteria.
- 2 In what ways do cyanobacteria differ from other phototrophic bacteria?
- 3 If cyanobacteria evolve oxygen, and if nitrogenase is oxygen-labile, how can cyanobacteria fix nitrogen?
- 4 How do the members of the order Myxobacterales differ from other gliding bacteria?
- 5 Besides the organisms listed under Gliding, Nonfruiting Bacteria, what other kinds of bacteria may exhibit gliding motility?



- 6 Prosthecae occur in bacteria other than those described in the section entitled Budding and/or Appendaged Bacteria. Give two examples.
- 7 Give an example of a genus of budding bacteria in which (a) the bud forms on a prostheca, (b) the bud forms directly on the mother cell.
- 8 How does "false branching" differ from "true branching"?
- 9 In what environments would one expect to find the following bacteria?
 

(a) <i>Halobacterium</i>	(d) <i>Sulfolobus</i>
(b) <i>Chromatium</i>	(e) <i>Gallionella</i>
(c) <i>Capnocytophaga</i>	(f) <i>Sphaerotilus</i>
- 10 What is the most significant difference between the following?
 

(a) <i>Methylococcus</i> (see Chap. 13) and <i>Methanococcus</i>
(b) <i>Rhodomicrobium</i> and <i>Hyphomicrobium</i>
(c) <i>Nitrococcus</i> and <i>Nitrosococcus</i>
(d) <i>Aquaspirillum</i> (see Chap. 13), <i>Rhodospirillum</i> , <i>Thiospirillum</i> , and <i>Methanospirillum</i>
- 11 List the features of archaeobacteria that distinguish them from eubacteria.
- 12 What property of *Halobacterium* might allow one to consider this genus as facultatively phototrophic? What differences exist between halobacteria and the phototrophic bacteria described in the first section of this chapter?
- 13 Define the following terms:
 

Bacteriorhodopsin	Akinete	Fruiting body
Phycobilins	Coenzyme F <sub>420</sub>	Swarm Cells
Thylakoids	Carotenoids	Thermoacidophile
Sporangiole	Heterocyst	Pseudomurein

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- Starr, M. P., H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (eds.): *The Prokaryotes: A Handbook of Habitats, Isolation, and Identification of Bacteria*, Springer-Verlag, New York, 1981. Although not primarily concerned with classification, this monumental reference work provides a wealth of descriptive information and illustrations concerning the various genera of bacteria.



## Chapter 16

# The World of Bacteria IV: Gram-Positive Filamentous Bacteria of Complex Morphology

**OUTLINE** *Bergey's Manual of Systematic Bacteriology, Volume 4*  
**Filamentous Bacteria that Divide in More Than One Plane**  
**Filamentous Bacteria That Form True Sporangia**  
***Streptomyces* and Similar Genera**  
**Additional Filamentous Bacteria Having Uncertain Taxonomic Placement**

Volume 4 of *Bergey's Manual* is devoted entirely to certain aerobic, Gram-positive bacteria which form structures similar to those that are characteristically found in the microscopic eucaryotic fungi. These structures include (1) a **mycelium** composed of branched filamentous hyphae, these generally being of much smaller diameter (approximately 1  $\mu\text{m}$ ) than the hyphae formed by microscopic fungi (5 to 10  $\mu\text{m}$  in diameter), and (2) asexual spores, which are termed **conidiospores** (or simply **conidia**) if they are naked, or **sporangiospores** if they are enclosed within a specialized sac (**sporangium**). The spores represent a major means of reproduction, since they are produced in large numbers, each capable of giving rise to a new organism. Although they are not heat-resistant, the spores are resistant to desiccation and aid survival of the species during periods of drought. The Gram-positive filamentous bacteria are mainly harmless soil organisms, although a few are pathogenic for humans, other animals, or plants. In the soil they are saprophytic and chemoorganotrophic, and they have the important function of degrading plant or animal residues; however, some are best known for their ability to produce a wide range of antibiotics useful in treating bacterial diseases. One genus fixes  $\text{N}_2$  symbiotically in woody plants. In this chapter we shall consider some of the genera included in the fourth volume of *Bergey's Manual*.

### BERGEY'S MANUAL OF SYSTEMATIC BACTERI- OLOGY, VOLUME 4

Table 16-1 lists the major groups of bacteria included in Volume 4 of the *Manual*. The reader should also note that some bacteria, the nocardioforms, are included in Volume 2 of the *Manual* rather than Volume 4 and have been described previously in this textbook (see Chap. 14). The dividing line between the no-



**Table 16-1.** Gram-Positive Filamentous Bacteria Included in *Bergey's Manual*, Volume 4

Section	Some Major Characteristics
FILAMENTOUS BACTERIA THAT DIVIDE IN MORE THAN ONE PLANE	The hyphae divide not only transversely but also longitudinally to produce clusters or packets of cells or spores; cell-wall type III*; soil organisms, animal pathogens, and symbiotic nitrogen-fixers are represented
FILAMENTOUS BACTERIA THAT FORM TRUE SPORANGIA	Harmless soil and water organisms whose hyphae divide in a single plane; the spores are formed within special sacs; cell-wall types II or III*
STREPTOMYCES AND SIMILAR GENERA	The hyphae divide in a single plane; long chains of conidiospores are formed at the tips of sporogenic hyphae; the organisms are mainly harmless soil organisms that are noted for production of antibiotics; a few are human or plant pathogens; cell-wall type I*
ADDITIONAL FILAMENTOUS BACTERIA HAVING UNCERTAIN TAXONOMIC PLACEMENT	A heterogeneous collection of organisms whose relationships to the major groups of Gram-positive filamentous bacteria is not yet agreed upon; some have remarkable morphological or physiological properties; a few organisms are pathogenic for humans; the cell-wall types vary

\* For cell-wall types, see Table 16-2.

cardioforms and the bacteria described in the present chapter is indistinct and controversial, and is based upon such factors as (1) whether or not the hyphae can reproduce by fragmentation and (2) the chemical composition of the cells, particularly the walls. In general, the organisms described in Volume 4 of the *Manual* do not undergo fragmentation of the mycelium. With regard to chemical composition, Table 16-2 indicates the major patterns that occur among the Gram-positive filamentous bacteria with regard to cell-wall amino acid composition

**Table 16-2.** Amino Acid and Sugar Patterns of Aerobic, Gram-Positive, Filamentous Bacteria

Composition of Peptidoglycan	Amino Acid Pattern Designations for Cell-Wall Types			
	I	II	III	IV*
Optical isomer of diaminopimelic acid present	LL	meso	meso	meso
Presence of glycine interpeptide bridges linking the tetrapeptides	+	+	-	-
Sugars Present in Hydrolysates of Whole Cells	Sugar Pattern Designations for Cell Types			
	A	B	C	D
Arabinose	+	-	-	+
Galactose	+	-	-	-
Xylose	-	-	-	+
Madurose	-	+	-	-

\*Type IV is distinguished from Type III by the occurrence of the sugars arabinose and galactose in the cell walls of Type IV.

and to the sugars present in whole cell hydrolysates; these differences, as well as differences in DNA base composition (mol % G + C values; see Chap. 3) and in the phospholipid composition of the cell membranes, have been used extensively to classify these organisms, in addition to the morphological features. For example, the genus *Streptomyces* has a type I cell-wall amino acid pattern and a type C sugar pattern (i.e., chemotype I, C), whereas *Dermatophilus* has a type III amino acid pattern and a type B sugar pattern (chemotype III, B). The bacteria included in Volume 4 of *Bergey's Manual* are mainly of the following types: I, C; II, D; III, B; or III, C, whereas the nocardioforms included in Volume 2 of the *Manual* are mainly type 4, A. Moreover, the organisms included in Volume 4 do not possess mycolic acids in their cell walls, whereas some nocardioforms do have these lipids.

### FILAMENTOUS BACTERIA THAT DIVIDE IN MORE THAN ONE PLANE

#### *Dermatophilus*

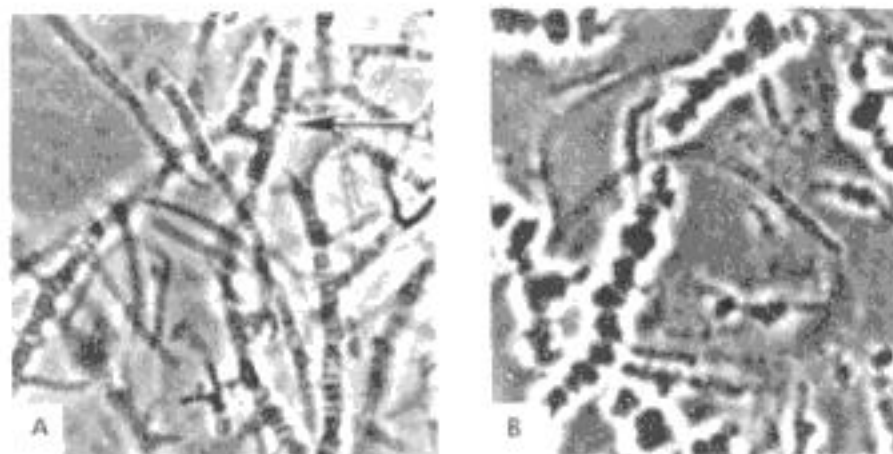
The organisms in this group have hyphae that divide not only transversely but also longitudinally to produce clusters or packets of coccoid or cuboid cells or spores. One of the three genera, *Geodermatophilus* (chemotype III, C) is a soil organism; the other two genera are quite different, as indicated below.

The members of this genus belong to chemotype III, B. The narrow tapering hyphal filaments develop septa that are formed in transverse and in horizontal and vertical planes; this results in the formation of mulberrylike clusters of cocci (see Fig. 16-1). Each coccus is released as a motile spore bearing a tuft of flagella. The single species of the genus, *D. congolensis*, is not a soil organism; rather, it is a parasite and pathogen of wild and domestic mammals, causing a skin infection (streptothrichosis).

#### *Frankia*

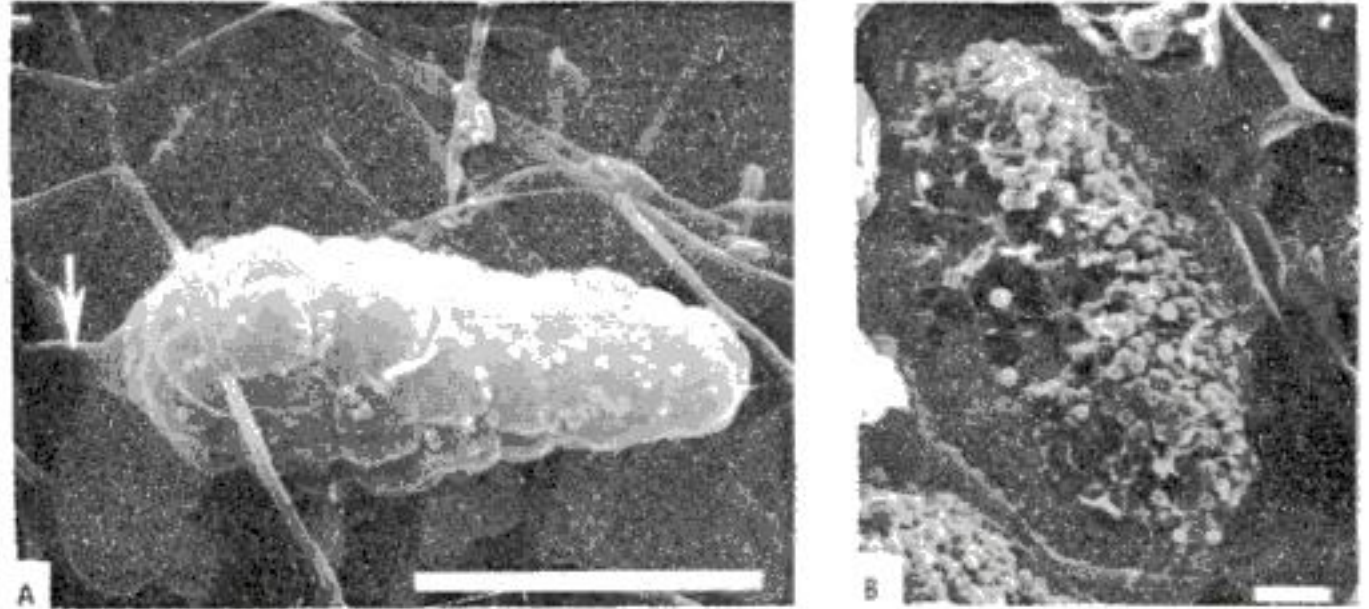
The members of this genus belong to Chemotype III, D. Like *Rhizobium* species

**Figure 16-1.** Phase-contrast photomicrographs of *Dermatophilus congolensis* suspended in a 28% albumin solution to reveal cellular detail (X1,100, approx.). (A) Branching hyphae in early stages of division that is mainly transverse, although a few longitudinal septa are already evident (arrow). (B) Mulberrylike clusters of cocci enveloped in mucoid capsular material that appears light against the denser, more refractive, albumin solution. [Courtesy of D. S. Roberts, in M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (eds.), *The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria*, Springer-Verlag, New York, 1981, p. 2011.]





**Figure 16-2.** (A) Scanning electron micrograph of a large sporogenic body of *Frankia* strain AvcII from broth culture; thickened spore-bearing hypha (arrow) attaches the spore-bearing body to a vegetative hypha. Bar indicates 10  $\mu$ m. (Courtesy of D. Baker and J. G. Torrey, *Can J Microbiol* 26:1066, 1980.) (B) Scanning electron micrograph illustrating numerous *Frankia* vesicles and hyphae within the infected cell of a root nodule of *Elaeagnus umbellata*. Bar indicates 10  $\mu$ m. (Courtesy of D. Baker, W. Newcomb, and J. G. Torrey, *Can J Microbiol*, 26:1072, 1980.)



(see Chap. 13), these bacteria are highly efficient microaerophilic  $N_2$ -fixers that occur within the root nodules of plants. Unlike *Rhizobium* species, however, they infect nonleguminous woody plants, such as alders. Isolation of the symbiont defied all efforts for years, but in 1978–1980 cultures were isolated by means of elaborate procedures such as (1) microdissection of nodules to remove the bacterial clusters, or (2) sucrose gradient centrifugation of nodule suspensions, which allowed separation of the organisms from extraneous material on the basis of differences in density. Both within root nodules and in laboratory culture, *Frankia* strains form branching hyphae from the ends of which are produced vesicles (globular structures on short stalks; see Fig. 16-2); circumstantial evidence suggests that these may be the sites of  $N_2$  fixation. Also produced are sporogenic bodies (often called sporangia, but whether they are true sporangia is uncertain) which contain masses of spores that are cemented together (Fig. 16-2). The spores arise by hyphal divisions that occur in several planes.

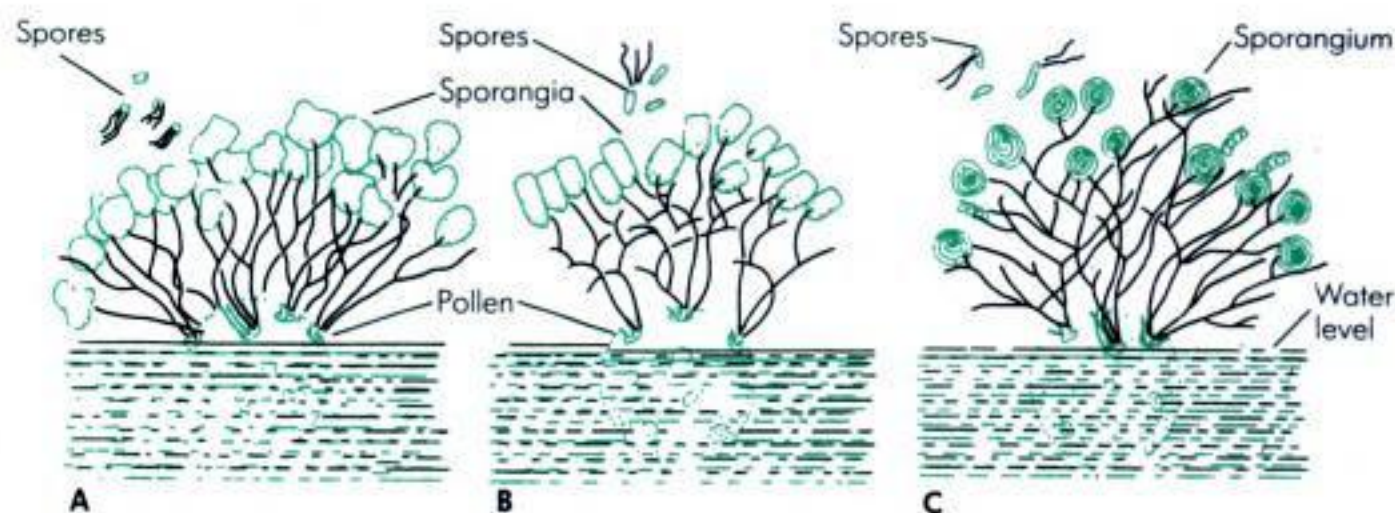
### FILAMENTOUS BACTERIA THAT FORM TRUE SPORANGIA

The members of this group form an extensive substrate mycelium; some genera also form an aerial mycelium. In either instance spores are formed within a sac called a sporangium, which is formed aurally at the tip of a sporogenic hypha (sporangiophore).

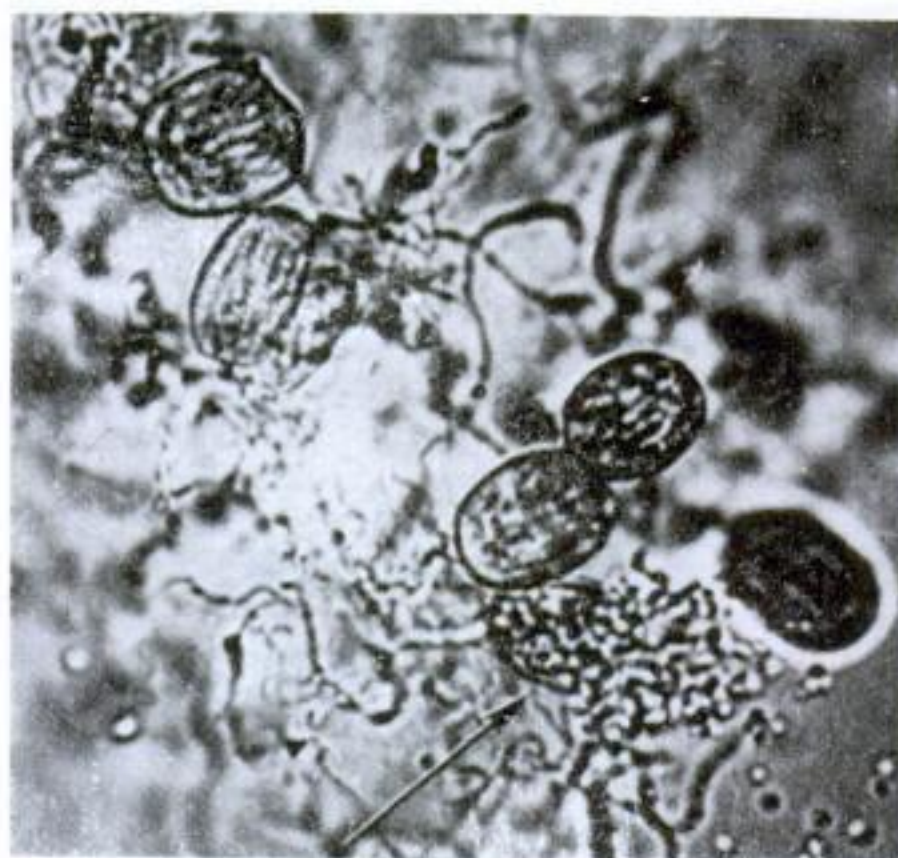
The sporangium may be either of two major kinds, depending on the genus: (1) round, rod-shaped, or irregular, containing masses of spores, or (2) fingerlike, club-shaped, or pear-shaped, containing one to four spores arranged linearly within the sporangium. The wall of the sporangium is derived from an expansion of the outer layer of the bilayered wall of the hypha, forming a sac. The inner hypha grows into this sac and subsequently undergoes septation to form spores. Some examples of genera forming multispored sporangia are illustrated in Figs. 16-3 and 16-4; in some instances the spores are motile by means of flagella and can swim away from ruptured sporangia (see Fig. 16-5). Chemotypes of sporangia-forming bacteria are either III, B or II, D. Sporangia-forming bacteria are widespread in nature, occurring in humus-containing soils, dead plant parts such as pollen and leaves, or on shed animal material such as hair.



**Figure 16-3.** Semidiagrammatic drawings of three genera of bacteria that form multisporous sporangia. The bacteria are growing on the pollen of *Liquidambar styraciflua*. The pollen floats at the surface of the water, the hyphae and sporangio-phores emerging into the air through the small circular pits in the wall of the pollen grains. (A) *Actinoplanes*. (B) *Ampullariella*. (C) *Spirillospora* (X350). (From McGraw-Hill Encyclopedia of Science and Technology, vol. 1, 1968.)



**Figure 16-4.** *Streptosporangium roseum*, showing sporangia, containing nonmotile spores, on aerial hyphae. The sporangia range from 7 to 19  $\mu\text{m}$  in diameter, usually 8 to 9  $\mu\text{m}$ . (Courtesy of Mary P. Lechevalier.)



**Figure 16-5.** Sporangia of *Actinoplanes rectilineatus* in a water mount showing spores in longitudinal rows within the sporangia and one ruptured sporangium (arrow) with spores swimming away (X970). (Courtesy of M. P. Lechevalier and H. A. Lechevalier, *Int J Syst Bacteriol*, 25:371, 1975.)



**Figure 16-6.** *Streptomyces viridochromogenes* showing spiral chains of spores on aerial mycelium. Specimen from culture grown on yeast extract-malt extract medium for 7 days at 25°C (X450). (Courtesy of Tom Cross.)



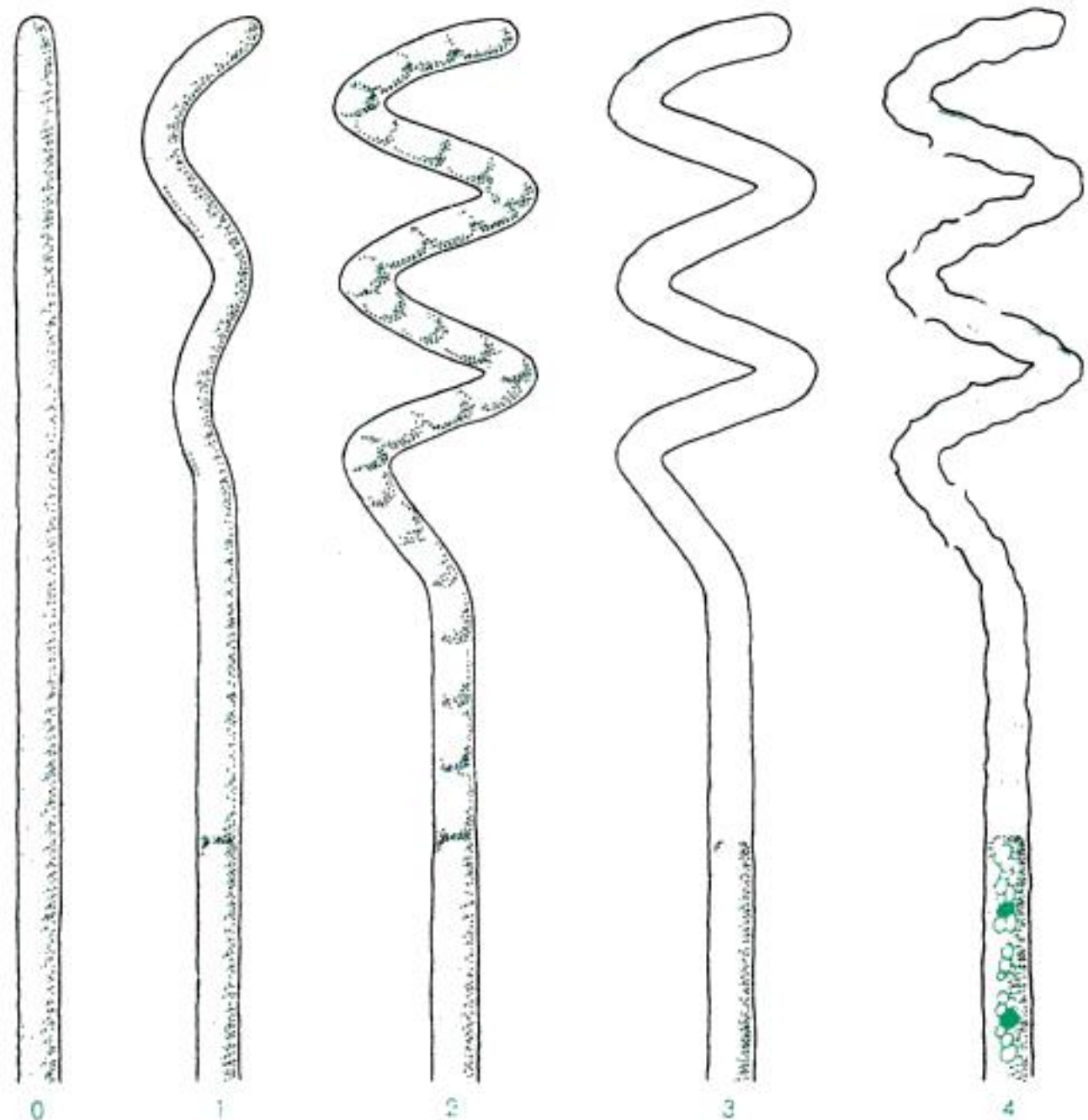
Species of the genus *Actinoplanes* can polymerize amino acids to form polypeptide and depsipeptide antibiotics and can also synthesize polyene-type macrolides, glycolipid, and aromatic polycyclic antibiotics.

## STREPTOMYCES AND SIMILAR GENERA

The members of this group belong to chemotype I, C. They usually form an abundant aerial mycelium bearing long chains of conidiospores (5 to 50 or more per chain; see Fig. 16-6). Unlike the hyphae of the substrate mycelium (i.e., submerged below the surface of the medium), the aerial hyphae possess an extra cell-wall layer (sheath). The hyphal tip undergoes septation within this sheath to form a chain of conidia (see Fig. 16-7).

Several genera occur within the group, including *Streptoverticillium*, *Actinopycnidium*, *Actinosporangium*, *Chainia*, *Elytrosporangium*, *Kitasatoa*, and *Microellobosporia*, but the most familiar genus is *Streptomyces*. Indeed, the other genera—except for *Streptoverticillium*—may deserve to be merged with *Streptomyces* to form a single genus. Species and subspecies are differentiated by the following characteristics:

**Figure 16-7.** Diagram of sporulation stages in *Streptomyces coelicolor*. After a phase of growth (0) the sporulating hyphae are divided into long cells by ordinary cross walls, and the tips begin to coil (1). The apex is then partitioned into spore-sized compartments by sporulation septa (2). The cell walls thicken and constrictions appear between the young spores (3). As spores mature, they round off and separate (4). Some spores begin to germinate immediately after maturation. (Redrawn from H. Wildermuth and D. A. Hopwood, *J Gen Microbiol*, 60:51–59, 1970; by permission.)





- 1 **Morphology of spore chain.** The chains may be straight, flexuous (wavy), or coiled to various degrees. They also vary in the number of spores per chain.
- 2 **Spore surface.** By electron microscopy, the surface of the conidia may be smooth, warty, spiny, or hairy, the texture depending on the kind of adherent sheath material (for example, see Fig. 16-8).
- 3 **Color of aerial mycelium.** The pigmentation of the aerial mycelium falls within any of seven color groups: white, yellow, violet, red, blue, green, or gray. These terms cannot precisely describe the many hues that can occur; therefore, a system of published color standards is used for this purpose.
- 4 **Color of substrate mycelium.** This may differ from that of the aerial mycelium and is determined by observing the reverse side of the growth after removal of most of the agar medium with a razor blade.
- 5 **Color of the medium.** Many streptomycetes may form pigments that are excreted into the medium. These may be water-soluble, or they may precipitate in the medium close to the cells.
- 6 **Physiological characteristics.** This refers to characteristics such as utilization of various carbohydrates and organic acids, nitrate reduction, urea and esculin hydrolysis.

No other genus in bacteriology has been divided into as many species as has *Streptomyces*. The names of nearly 340 species and 39 subspecies are officially recognized, but thousands of additional names have been unofficially assigned (mostly in literature dealing with patents; see below). It is likely that DNA homology and other techniques of molecular biology will eventually indicate that a much smaller number of species exists than is represented by these names. Why then have so many names been used? The main reason rests with the ability of streptomycetes to make a great number and variety of antibiotics (see Table 16-3 for a few examples). As a result of the screening of soil samples by pharmaceutical companies to obtain new antibiotics, thousands of new antibiotics have been discovered, and the great majority have been made by streptomycetes. The discoverer of a new antibiotic had to have a name for the organism producing it in order to meet scientific publication or patent requirements. The organism might have been identified as belonging to an established species; however, many isolates failed to agree in all details with the description of any established species. Although some variation in characteristics does occur among the strains of any bacterial species, an easier (but much less satisfactory) solution to such a taxonomic problem was simply to give the isolate a new

**Figure 16-8.** Hairy conidia of *Streptomyces acrimycini* as seen by (A) transmission electron microscopy and (B) scanning electron microscopy. The bar indicates 1  $\mu\text{m}$ . (Courtesy of A. Dietz and J. Mathews, *Int J Syst Bacteriol*, 27:282, 1977.)







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production of certain antifungal antibiotics, how might streptomycetes be able to compete successfully with fungi when growing in soil?

- 7 Despite its extreme halophilic nature, *Actinopolyspora* belongs to the eubacteria and not to the archaeobacteria (in contrast, for example, to *Halobacterium*). What sort of evidence might have led to this conclusion?
- 8 Define the following terms: synnemata, conidia, sporangiophore, sessile spore, sporangium, chemotype.

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Williams, S. T. (ed.): *Bergey's Manual of Systematic Bacteriology*, vol. 4, Williams & Wilkins, Baltimore (in preparation). Volume 4 of this international work is presently being prepared and will provide detailed descriptions of the genera and species of the bacteria discussed in Chap. 16.





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## The many kinds of microbes

Professor John O. Corliss of the University of Maryland is recognized for his scholarly knowledge of the protozoa. His recent contribution entitled "A Puddle of Protists" (in *The Sciences*, New York Academy of Science, May-June, 1983) is recommended reading to introduce this part of the text. And so in the discourse that follows, we borrow freely from his article the historical development of human knowledge on the protists and other "wee beasties."

It all began in the nineteenth century when the German evolutionist Ernst Haeckel proposed a third biological kingdom, the Protista, to include all the many microbes, large and small, especially those which could not be neatly classified with either the plants or the animals. While this was a convenient taxonomic device, it had its own problems because the microbes assembled together included members which were fundamentally dissimilar. For instance, the bacteria, the protozoa, the algae, and the fungi were all lumped together. This is not to say that there was no common thread running through them all; indeed, to be counted as a protist, an organism must be unicellular in at least one stage of its life history and must at no stage develop organized tissues. Nonetheless, Haeckel's revolutionary proposal essentially fell flat and has laid dormant for all these years.

After Haeckel, the next breakthrough in the classification of the largely unicellular forms came a century later. Roger Y. Stanier and his colleagues at the University of California at Berkeley proposed two assemblages of organisms based mainly on the type of nucleus pres-

ent within their cells. These two kingdoms of organisms are called the procaryotes (prenucleated cells) and the eucaryotes (cells with a true nucleus). The number of their differential characteristics (some of which we have propounded in the beginning of this text) has grown as biologists probe into the molecular properties of the cells of these two groups. Such findings have strengthened the major dichotomy between them.

Very recently, Carl Woese, of the University of Illinois, and other molecular geneticists working primarily with bacteria, have proposed that the procaryotes themselves should be split into two groups. This split results in three kingdoms of organisms: the **Eucaryota**, the **Archaeobacteria** of Woese, and the rest of the **Procaryota**. The archaeobacteria are distinguished from the other procaryotes by the sequence of bases in their ribosomal RNA, the chemical composition of their lipids, and their cell wall structure. They constitute a group of procaryotes that are found in specialized or extreme habitats—the hot acidic niches of the thermoacidophilic bacteria, the nearly saturated salt solutions of the extreme halophilic bacteria, and the highly anaerobic environment required for the growth of the methane-producing bacteria.

In this part of the text we shall look at the world of the eucaryotic protists—the fungi (molds and yeasts), the algae, the protozoa—as well as that group of taxonomically elusive infectious entities which we call the viruses and which we are hard pressed to know where to place in the evolved classification schemes for all the life forms.



*image  
not  
available*

## THE IMPORTANCE OF FUNGI

The fungi are heterotrophic organisms—they require organic compounds for nutrition. When they feed on dead organic matter, they are known as **saprophytes**. Saprophytes decompose complex plant and animal remains, breaking them down into simpler chemical substances that are returned to the soil, thereby increasing its fertility. Thus they can be quite beneficial to humans. But they can also be undesirable when they decompose timber, textiles, food, and other materials.

Saprophytic fungi are also important in industrial fermentations: for example, the brewing of beer, the making of wine, and the production of antibiotics such as penicillin. The leavening of dough and the ripening of some cheeses also depend on fungal activity.

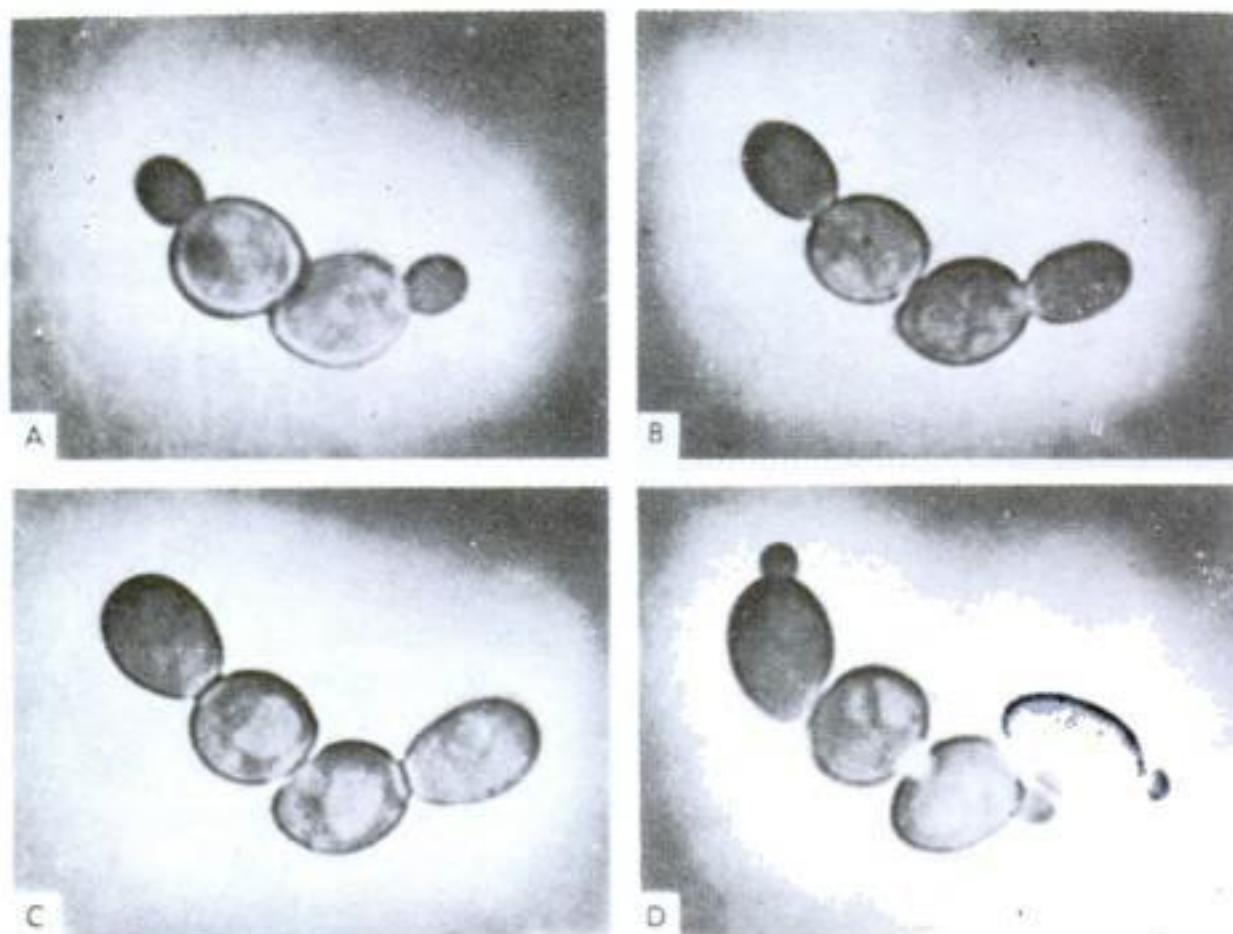
As **parasites** (i.e., when living in or on another organism), fungi cause diseases in plants, humans, and other animals. Although fungal diseases are less commonly encountered than bacterial or virus diseases in humans and other animals, they are of great importance in causing diseases of plants.

Quite apart from the applied aspects of the study of fungi, these microorganisms are to be studied in their own right as biological entities. The science or study of fungi is called **mycology**. Fungi have also become tools for the physiologist, biophysicist, geneticist, and biochemist, who find them highly suitable subjects for the study of some biological processes.

## DISTINGUISHING CHARACTERISTICS OF FUNGI

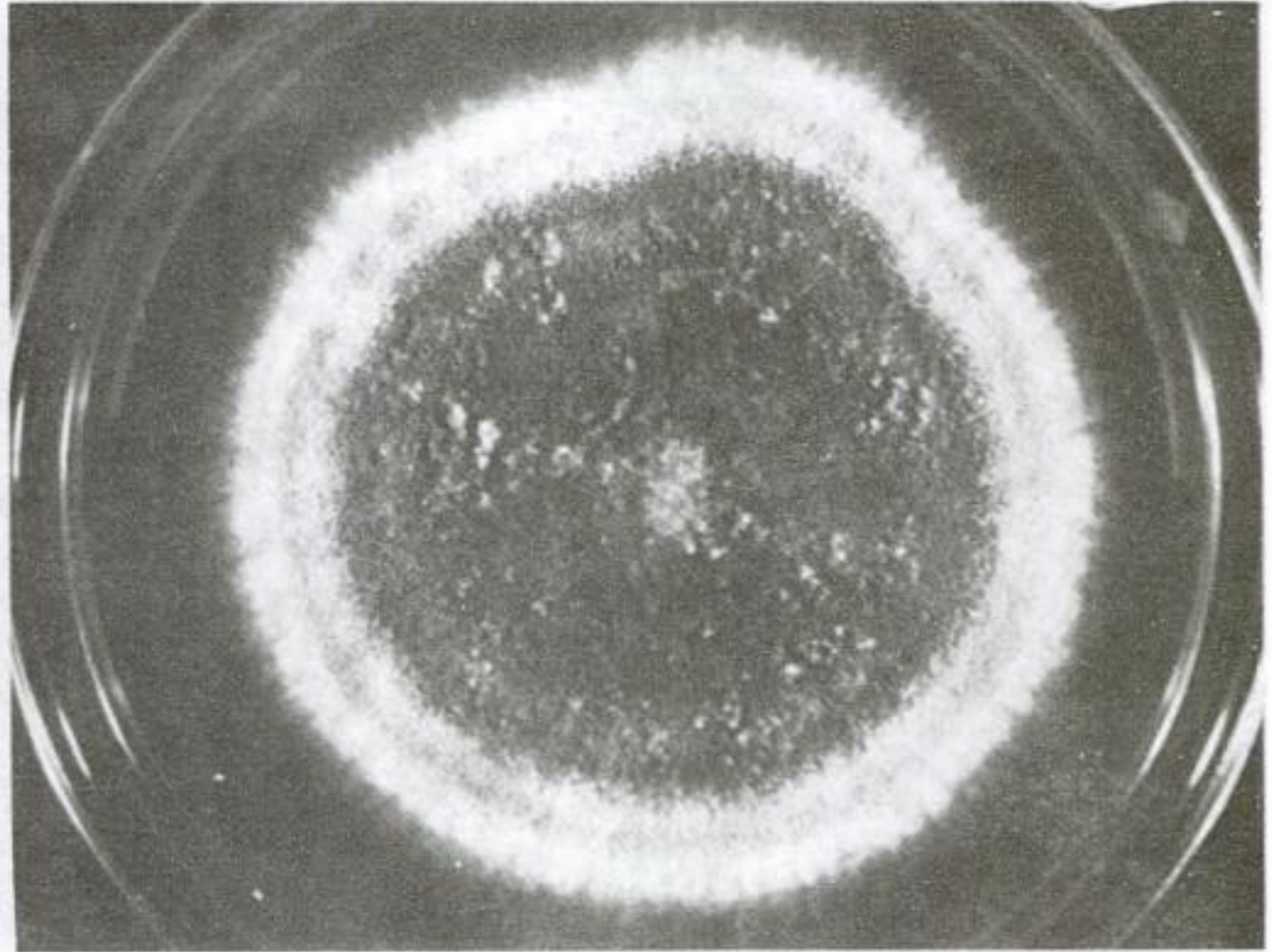
Fungi are eucaryotic chemoorganotrophic organisms that have no chlorophyll. The **thallus** (plural, **thalli**), or body of a fungus may consist of a single cell as in the yeasts; more typically the thallus consists of filaments, 5 to 10  $\mu\text{m}$  across, which are commonly branched. The yeast cell or mold filament is surrounded by a true cell wall (the exception being the slime molds, which have a thallus

**Figure 17-1.** Actively budding yeasts produce mature daughter cells in about 30 min. These photographs were taken at 10-min intervals. The typical unicellular nature of yeast cells is shown. (Courtesy of Carl C. Lindegren.)





**Figure 17-2.** A mold colony growing in a Petri dish. Note the filamentous growth of the organism. The powdery appearance is due to the presence of thousands of asexual spores, or conidia. The species shown belongs to the genus *Penicillium*, the same genus of mold that produces the antibiotic penicillin.



consisting of a naked amoeboid mass of protoplasm). Fig. 17-1 shows the typical morphology of a yeast cell.

Some fungi are **dimorphic**; that is, they exist in two forms. Some pathogenic fungi of humans and other animals have a unicellular and yeastlike form in their host, but when growing saprophytically in soil or on a laboratory medium they have a filamentous mold form. The laboratory identification of such fungal pathogens is often dependent on the demonstration of dimorphism. The opposite dimorphic phenomenon occurs in some plant pathogens. In *Taphrina* (which causes peach leaf curl) or in smuts (which cause diseases of cereal crops), the mycelial form occurs in the host and the unicellular yeastlike form occurs in laboratory culture.

Thus a fungal colony may be a mass of yeast cells (not unlike a bacterial colony except for surface texture), or it may be a filamentous mat of mold as shown in Fig. 17-2.

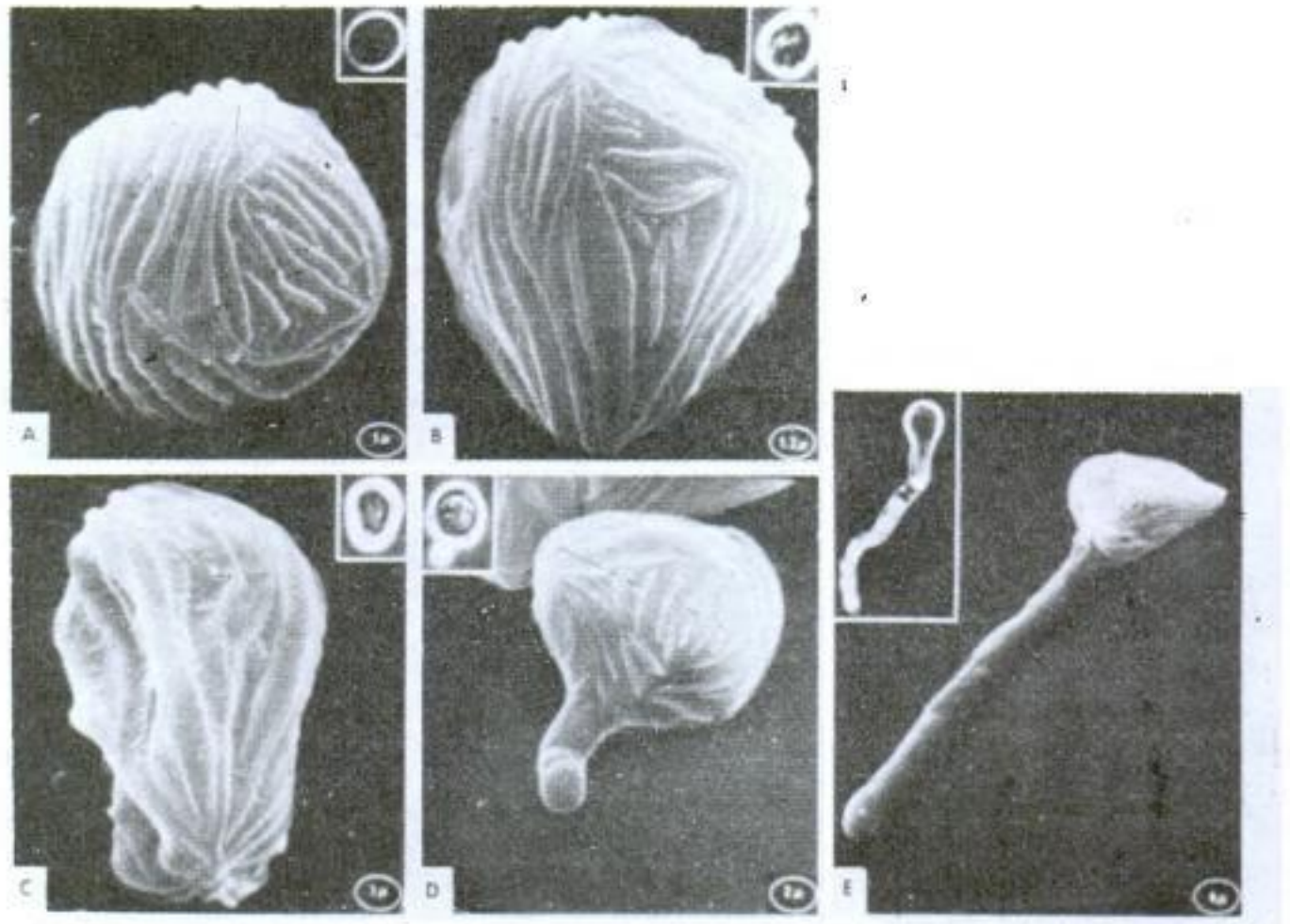
## MORPHOLOGY

In general, yeast cells are larger than most bacteria. Yeasts vary considerably in size, ranging from 1 to 5  $\mu\text{m}$  in width and from 5 to 30  $\mu\text{m}$  or more in length. They are commonly egg-shaped, but some are elongated and some spherical. Each species has a characteristic shape, but even in pure culture there is considerable variation in size and shape of individual cells, depending on age and environment. Yeasts have no flagella or other organelles of locomotion.

The thallus of a mold consists essentially of two parts: the **mycelium** (plural, **mycelia**) and the **spores** (resistant, resting, or dormant cells). The diversity of



**Figure 17-3.** Scanning electron micrographs of *Rhizopus stolonifer* spores at sequential stages of germination, with corresponding phase-contrast micrographs (X1,200) inserted. (A) Ungerminated spore; (B) swollen spore; (C) elongated spore; (D) germ-tube emergence; and (E) germ-tube elongation. (Courtesy of James L. van Etten, Lee A. Bulla, Jr., and Grant St. Julian, "Physiological and Morphological Correlation of *Rhizopus stolonifer* Spore Germination," *J Bacteriol*, 117:882–887, 1974.)



these spores will be discussed later. The mycelium is a complex of several filaments called **hyphae** (singular, **hypha**). New hyphae generally arise from a spore which on germination puts out a germ tube or tubes (Fig. 17-3). These germ tubes elongate and branch to form hyphae.

Each hypha is about 5 to 10  $\mu\text{m}$  wide, as compared with a bacterial cell which is usually 1  $\mu\text{m}$  in diameter. Hyphae are composed of an outer tubelike wall surrounding a cavity, the **lumen**, which is filled or lined by protoplasm. Between the protoplasm and the wall is the **plasmalemma**, a double-layered membrane which surrounds the protoplasm. The hyphal wall consists of microfibrils composed for the most part of hemicelluloses or chitin; true cellulose occurs only in the walls of lower fungi. Wall matrix material in which the microfibrils are embedded consists of proteins, lipids, and other substances.

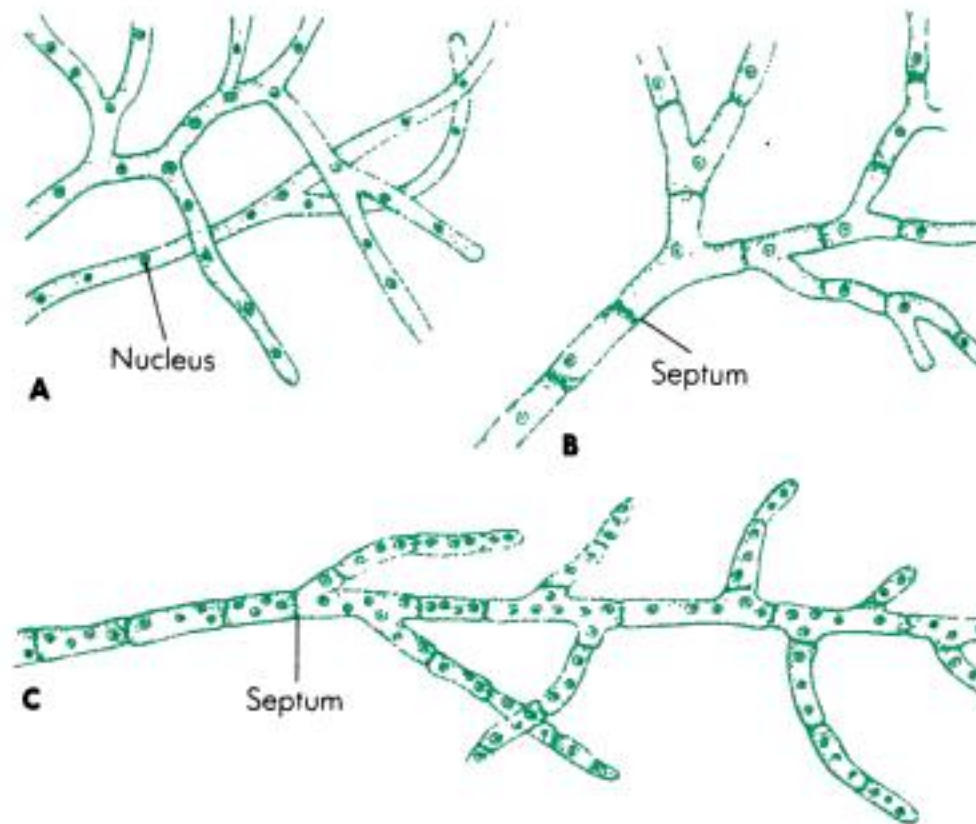
Growth of a hypha is distal, near the tip. The major region of elongation takes place in the region just behind the tip. The young hypha may become divided into cells by crosswalls which are formed by **centripetal invagination** (inward growth) from the existing cell wall. These crosswalls constrict the plasmalemma and grow inward to form generally an incomplete **septum** (plural, **septa**) that has a **central pore** which allows for protoplasmic streaming. Even nuclei may migrate from cell to cell in the hypha.

Hyphae occur in three forms (Fig. 17-4):

- 1 Nonseptate, or **coenocytic**. Such hyphae have no septa.
- 2 Septate with uninucleate cells.
- 3 Septate with multinucleate cells. Each cell has more than one nucleus in each compartment.

Mycelia can be either vegetative or reproductive. Some hyphae of the vege-





**Figure 17-4.** Three types of hyphae. (A) Nonseptate (coenocytic), (B) septate with uninucleate cells, (C) septate with multinucleate cells.

tative mycelium penetrate into the medium in order to obtain nutrients; soluble nutrients are absorbed through the walls. (Insoluble nutrients are first digested externally by secreted enzymes.) Reproductive mycelia are responsible for spore production and usually extend from the medium into the air. The mycelium of a mold may be a loosely woven network or it may be an organized, compact structure, as in mushrooms.

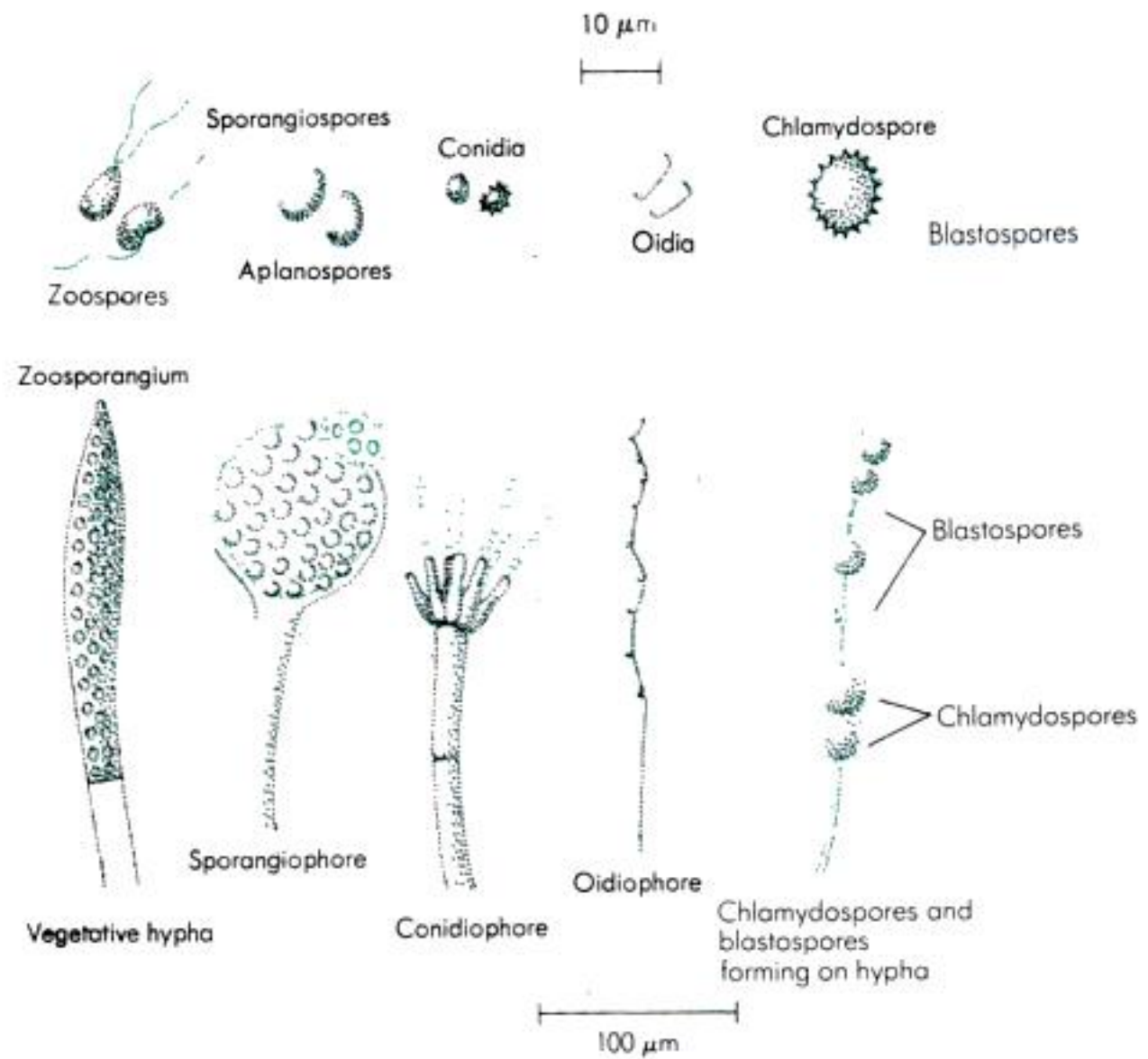
## REPRODUCTION

### Asexual Reproduction

Fungi reproduce naturally by a variety of means. Asexual reproduction (also called **somatic** or **vegetative reproduction**) does not involve the union of nuclei, sex cells, or sex organs. It may be accomplished by (1) fission of somatic cells yielding two similar daughter cells; (2) budding of somatic cells or spores, each bud a small outgrowth of the parent cell developing into a new individual; (3) fragmentation or disjoining of the hyphal cells, each fragment becoming a new organism; or (4) spore formation.

Asexual spores, whose function is to disseminate the species, are produced in large numbers. There are many kinds of asexual spores (Fig. 17-5):

- 1 **Sporangiospores.** These single-celled spores are formed within sacs called **sporangia** (singular, **sporangium**) at the end of special hyphae (**sporangiophores**). **Aplanospores** are nonmotile sporangiospores. **Zoospores** are motile sporangiospores, their motility being due to the presence of flagella.
- 2 **Conidiospores** or **conidia** (singular, **conidium**). Small, single-celled conidia are called **microconidia**. Large, multicelled conidia are called **macroconidia**. Conidia are formed at the tip or side of a hypha (see Fig. 17-6).
- 3 **Oidia** (singular, **oidium**) or **arthrospores**. These single-celled spores are formed by disjoining of hyphal cells. (See Fig. 17-7.)
- 4 **Chlamydospores.** These thick-walled, single-celled spores are highly resistant to adverse conditions. They are formed from cells of the vegetative hypha.
- 5 **Blastospores.** These are spores formed by budding.



**Figure 17-5.** Asexual spore types in fungi. (Redrawn from the McGraw-Hill Encyclopedia of Science and Technology, 1977, vol. 5, p. 117.)



**Figure 17-6.** Conidia are produced in large numbers as exemplified here by a species of *Penicillium* (X1,000). (Courtesy of Douglas F. Lawson.)





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